Investigating the cost of adaptation in *Amaranthus tuberculatus* populations with evolved resistance to glyphosate

Helen Maria Cockerton

A thesis for the degree of Doctor of Philosophy

Submitted to the School of Life Sciences, University of Warwick

September 2013

Contents

List of figures

List of tables

Acknowledgements

Primarily, I would like to thank my supervisor Dr. Paul Neve. I greatly appreciate the level of guidance and feedback Paul has provided throughout the course of this project. Paul's scientific rigor and questioning has led me to establish a greater understanding of the work I have conducted. I would also like to thank my supervisor Prof. Deepak Kaundun for his support and for sharing much of his extensive knowledge with me. Both Deepak and Paul have been engaging and helpful supervisors who have made the undertaking of this project interesting and enjoyable.

Furthermore, I wish to express my gratitude to the people that have helped me throughout my Ph. D: Ian Zelaya for the supervision provided in the initial stages of this project, Sarah-Jane Hutchings for her guidance on dose response experiments, Richard Dale for his training in molecular procedures and Anushka Howell for sharing with me her substantial experience in bio-kinetics. Thanks are also due to Andrew Mead for his statistical advice and Peter Watson for accommodating all of my plant production requests.

I am extremely grateful to the Neve group, horticultural services (past and present) and others who have helped me with large experimental set ups. In particular my appreciation goes to Tony Carter, Laura Davies and John Lynch for engaging in discussions regarding aspects of our research areas.

I acknowledge the financial support provided by Syngenta and BBSRC.

I would like to thank all of my office mates and house mates over the years for making the time I have spent at HRI and in Leamington Spa enjoyable.

Massive amounts of gratitude go to Andy, not only for the helpful scientific discussions but also for being supportive, friendly, making me laugh and generally being awesome.

Finally, I thank my mum and dad, and my siblings; Faith, Michael and Sophie for their support and encouragement over the last four years.

Declaration

This thesis is presented in accordance with the regulations for the degree of Doctor of Philosophy. It has been composed by myself and has not been submitted in any previous application for any degree. The work in this thesis has been undertaken by myself except where otherwise stated.

Summary

Amaranthus tuberculatus (Common Waterhemp) is a prevalent, problematic weed in Midwestern USA, where genetically-modified crops are widely grown, resulting in multiple annual applications of glyphosate. Such practices provide the selection pressure for the evolution of glyphosate resistance. Evolutionary theory predicts that adaptation to novel stresses, such as herbicide application, will incur a cost in the original (herbicide-free) environment. This project aims to identify whether glyphosate resistance in a population of *Amaranthus tuberculatus* was associated with a fitness cost in the absence of glyphosate. Initial dose response experiments on the study population determined a resistance index of 3.2 compared to a standard sensitive population. To generate appropriate material for comparisons between phenotypes, individual plants were cloned and their resistance status determined by glyphosate application. Parent plants were designated as resistant or susceptible and appropriate crosses were performed to generate seed lines. The incomplete segregation of resistant and susceptible seed lines indicated that resistance was controlled by a quantitative trait. *EPSPS* gene amplification was elucidated as the primary mechanism of glyphosate resistance. There was a strong positive relationship between half-sibling seed family LD_{50} and relative *EPSPS* gene copy number indicating that resistance was primarily caused by target-site gene amplification. Subsequent fitness experiments used seed families to determine that no fitness trade-off was associated with resistance (or gene copy number) when plants are grown without competition, in glasshouse and polytunnel growth environments. However, a small growth penalty was associated with elevated gene copy number in plants grown in a polytunnel, without competition (10% biomass reduction at +20 *EPSPS* copies), this led to competition experiments. A fitness cost was associated with resistance under intra-phenotypic competition; however, the cost was mitigated under inter-specific competition with maize. A cost was associated with glyphosate resistance and this cost was influenced by interactions with biotic and abiotic factors.

1.0 Literature review

1.1 The Impact of weeds and global weed control trends

By 2050, the world population is predicted to rise from 7 to 9.1 billion people (United Nations, 2009). Furthermore, the demand for food is predicted to increase disproportionally, with a 70% increase in demand worldwide between 2005 and 2050 (FAO, 2009; Balmford *et al.*, 2012). This figure is impacted not only by the increase in the global population but also by the increase in per capita income of growing nations. It is therefore important to sustain and enhance crop yields so that the demand of the prospective world population is met. The maintenance of food security requires that scientists continue to conduct research into all areas of crop enhancement and protection. The area of crop protection with which this thesis is concerned is weed management. Weed management research is an area that requires continued investigation due the ability of weeds to evolve adaptations that result in the nullification of weed management practices.

1.1.1 Weed mediated reduction in crop yield

There are multiple agronomic strategies which are used to maximise crop yield. Primarily, the manipulation of crop genetics through conventional breeding and transgenic manipulation can enhance the absolute potential yield of a crop. Subsequently, the utilisation of agronomic practices such as fertilisation, allows the maximum potential yield of a crop to be achieved. Finally, the application of crop protection products helps minimise the potential yield loss that results from the invasion of pests, infection from diseases and competition from weeds (Oerke,

2006). Indeed, it was estimated that the impact of weed competition, in the absence of herbicide application, would have decreased the US economy by $20 billion in 1994 (Bridges, 1994). With respect to crop yield losses, weeds are a farmer's worst pest. Between 2001 and 2003, *Triticum aestivum* (wheat), *Oryza sativa* (rice), *Zea mays* (maize), *Solanum tuberosum* (potato), *Glycine max* (soya bean) and *Gossypium hirsutum* (cotton) yields would have suffered an estimated global potential crop loss of 23.0 %, 37.1 %, 40.3 %, 30.2 %, 37.0 % and 35.9 %, respectively, in the absence of weed control (Oerke, 2006). This loss is substantial when compared to the average global potential crop losses suffered as a result of uncontrolled herbivores (18%) and disease (16%) (Oerke, 2006). This comparatively large, weed mediated potential loss can be attributed to the fact that weeds are a chronic problem. Specifically, unlike herbivores and pathogens, the persistence and ubiquitous nature of weeds means that they are able to reduce crop yield throughout the growing season. Fortunately, the implementation of management strategies can mitigate weed mediated yield losses. Thus, the actual weed mediated crop yield reduction is between 7.5 and 10.5 % depending upon the crop. This 74% overall reduction in actual crop loss is due to the high efficacy of weed control measures (figure 1.1) (Oerke, 2006).

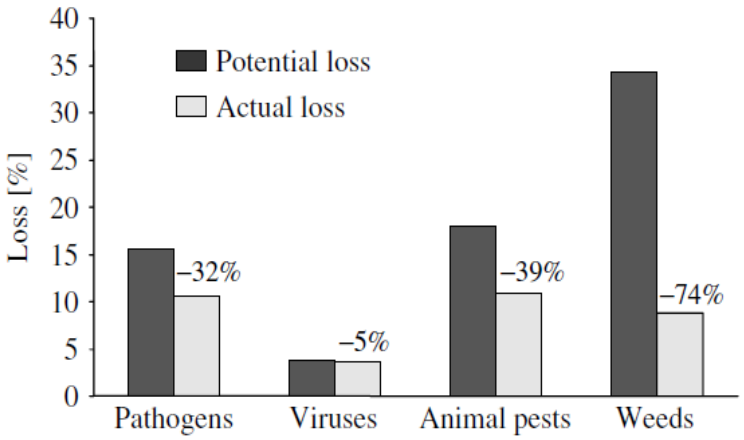


Figure 1.1: The estimated efficacy of management practices at mitigating the yield loss reduction caused by pathogens, viruses, animal pests, and weeds. The figures show the percentage change between potential and actual yield loss. (Source: Oerke, 2006).

1.1.2 Herbicides

Before the introduction of herbicides, weed control was achieved through human labour (hand weeding) and subsequently by mechanical and physical methods using horse- and then diesel-powered implements (Ghersa *et al.*, 2000b). Chemical herbicides were first introduced during the 1950's. Herbicide application results in the suppression and mortality of weeds, which ultimately reduces weed mediated crop yield losses. Such herbicide applications are normally supported by the use of existing weed management strategies and thus allow drastic improvements in weed control efficacy and costs, whilst reducing labor inputs (Bridges, 1994; Ghersa *et al.*, 2000b; Gianessi, 2007).

Principally, the crop production system found in Midwest USA will be discussed during this review as it is a highly relevant system to the study species. Herbicide application has become the primary form of weed control within the US (Gianessi & Reigner, 2007). Indeed, if the USA switched from the current system of herbicide application, to a tillage and hand weeding removal strategy, a total of 70 million labourers would be required and still there would be a 20% reduction in total crop yield (Gianessi & Reigner, 2007). This study not only highlights the nation's current reliance upon herbicides but also the necessity to safeguard herbicide efficacy and thus maintain the benefits associated with herbicide application.

Inevitably, the continuous utilization of herbicides has led to the evolution of herbicide resistant weed populations (Mortimer & Maxwell, 1994). This has greatly reduced the efficacy of many herbicides and minimised the number of options available for weed control. The evolution of herbicide resistance is the next escalation in the weed management-evolutionary arms race (Neve *et al.*, 2009). Thus far, 25 distinct groups of herbicides have been developed, each group forms a distinct chemical class with a specific mode-of-action and target-site (Duke, 2012). However, no herbicidal chemical class portraying a novel mode-of-action has been successfully launched globally since the HPPD introduction in 1991 (Rüegg *et al.*, 2007; Duke, 2012). Additionally, the development and rapid uptake of glyphosate tolerant crops has reduced the market for new herbicidal modes-of-action and thus also the efforts of herbicide discovery (Duke, 2012). The evolution of glyphosate resistant weeds has once again reintroduced the necessity for new herbicidal modes-of-action (Duke, 2012). Indeed, the rate of novel herbicide development is impeded by the monetary costs associated with complying to the strict environmental and

toxicological regulations required for the introduction of a new pesticide (Rüegg *et al.*, 2007). In addition to low herbicides discovery rates, the pool of herbicides available for use is decreasing, as many existing herbicides are being decommissioned and thus removed from use. This decommissioning is due to an increase in environmental and human health regulations, particularly within the EU as opposed to the USA (Rüegg *et al.*, 2007). It is important to maintain the efficacy of existing herbicides due to the deficit in novel herbicidal modes-of-action and the decommissioning of existing herbicides (Moss, 2010; Beckie & Tardif, 2012).

1.2 *Amaranthus tuberculatus (var. rudis)*

1.2.1 The evolution of A. tuberculatus (var. rudis)

A. tuberculatus (*var. rudis*) is considered to be a relatively new species. Initially, *Amaranthus rudis* (Common waterhemp) and *Amaranthus tuberculatus* (Tall waterhemp) occupied two independent territories and were considered to be two distinct species (Pratt & Clark, 2001; Trucco *et al.*, 2009). However, *A. rudis* migrated north and east from its original habitat in Oklahoma to overlap with the stationary, marsh range of *A. tuberculatus* (Sauer, 1957; Sauer, 1972; Trucco *et al.*, 2009). This allowed the two species to interbreed and to produce *A. rudis*- *A. tuberculatus* hybrids. The hybrids were termed *A. tuberculatus* (*var. rudis*) and were found in agricultural fields as weeds in 1957 (Sauer, 1957) indicating that the hybrids had an advantage over parental individuals for occupying such a niche. Before this point neither *A. rudis* nor *A. tuberculatus* were considered to be detrimental agricultural weeds (Trucco *et al.*, 2009). Over the last 23 years *A.*

tuberculatus (var. rudis) has evolved to become the principal problematic weed in Midwestern USA, particularly within corn and soybean production (Steckel, 2007)

Some people still report that unique morphological differences can be distinguished between *A. rudis* and *A. tuberculatus* indicating that full introgression of the two species has not occurred (Steckel, 2007). However, the overlapping ranges and frequent hybridisation of *A. rudis* and *A. tuberculatus* prevents the distinct classification of species (Pratt & Clark, 2001), therefore the experimental species has been described utilising the collective taxon of *A. tuberculatus*.

1.2.2 Biology

1.2.2.1 Seed biology

The seedling emergence within a single *A. tuberculatus* seed cohort in a natural system, was 39, 28, 10 and 0.004 % of the original seed bank every year over 4 years, respectively (Steckel *et al.*, 2007). These findings indicate a 77 % total seed viability and an approximate longevity of a seed cohort of up to 4 years (Steckel *et al.*, 2007). *A. tuberculatus* seed dormancy is phytochrome regulated and may be maintained by far red light and released by red light after a period of cold imbibing (Leon & Owen, 2003). This phytochrome regulated dormancy strategy promotes shade avoidance by larger plants.

The increasing incidence and abundance of *A. tuberculatus* as a major weed in Midwestern crop production systems has been facilitated by increased adoption of conservation and no-tillage systems (Owen & Zelaya, 2005; Steckel *et al.*, 2007). Tillage is an effective control strategy that significantly reduces *A. tuberculatus*

emergence (Steckel *et al.*, 2007). Indeed, a no-tillage crop production systems favours small seeded species such as *A. tuberculatus* as it maintains seed near the soil surface and thus promotes germination (Buhler, 1992).

A. tuberculatus has evolved yet more strategies to evade weed control attempts, it has become a particularly problematic weed due to the delayed emergence of seedlings (Uscanga-Mortera *et al.*, 2007). Seedling germination occurs from the end of April to July (Steckel *et al.*, 2007) and this results in the emergence of weed seed throughout the crop growing season (Hartzler *et al.*, 1999). This temporal escape tactic allows seedlings to establish after the initial post-emergence herbicide applications and therefore several weed management events are required throughout the crops life span.

1.2.2.2 Growth and competition

Problematic weeds have a propensity for fast growth which leads to intense crop competition; *A. tuberculatus* can reach between 2 to 3 m tall and has a rapid growth rate at an average of 0.135 cm of growth per growing degree day with a relative growth rate of 0.31 g g^{-I} day^{-1} (Horak & Loughin, 2000; Steckel, 2007). To place these figures into perspective, when compared to *A. palmeri, A. retroflexus* and *A. albus,* 3 major *Amaranthus* crop weeds, only *A. palmeri* surpasses *A. tuberculatus'* vigorous relative growth rate and growth per degree day (Horak & Loughin, 2000).

Weed mediated crop yield reduction is the ultimate negative consequence resulting from a weed infestation and thus yield reduction can be used to measure the detrimental impact of a weed population. The following studies indicate the degree

to which *A. tuberculatus* impacts upon crop yield. *A. tuberculatus* (removed 10 weeks after establishment), reduced soybean yield by an average of 43% (Hager *et al.*, 2002). An alternate study, found soybean yield was reduced by 56.2% *G. max* at a density of 8 *A. tuberculatus* plants m^{-2}, when competition occurred from crop emergence until harvest (Bensch *et al.*, 2003). Even the competitive impact of late emerging *A. tuberculatus* seed can result in a 10% reduction in *soybean* yield, indicating that weed management needs to be maintained throughout crop production to achieve potential yields (Bensch *et al.*, 2003). Similarly, *Z. mays* yield can be reduced from 11 to 74 % as a result of season-long competition with *A. tuberculatus*, this disparity in the impact of *A. tuberculatus* on *Z. mays* yield was attributed to variation in precipitation. It is interesting that environmental conditions were found to have a large impact upon yield whereas crop yield reduction was not found to vary depending upon weed density (Steckel *et al.*, 2004). Hager et al. (2002) reported that 180 *A. tuberculatus* plants m^{-2} could be found in uncontrolled fields. When considering a 56.2% yield reduction was observed in soybean at 8 plants m^{-2} (Bensch *et al.*, 2003), it is clear that *A. tuberculatus* will dramatically reduce soybean yields in uncontrolled weed fields. Evidently, effective weed management is essential in *A. tuberculatus* infested fields. Further to yield reduction, *A. tuberculatus* infestations can also result in complications within a field due to the interference with crop harvesting equipment (personal observation of Steckel, 2007).

1.2.2.3 Habitat

A. tuberculatus is one of twelve *Amaranthus* species (pigweeds) found in USA agriculture, all of which are summer annual weeds. *A. tuberculatus* is distributed

throughout the majority of North American states and the southern regions of Canada (figure 1.2). It can be found infesting corn, soybean, alfalfa and sorghum fields (Bensch *et al.*, 2003; Steckel & Sprague, 2004; DEFRA;Tatnell *et al.*, 2007). Notably, *A. tuberculatus* predominates the weed communities in most Midwestern USA states where it is a highly problematic weed (Wax, 1995 cited in Horak & Loughin, 2000). The predominance of *A. tuberculatus* has resulted from the selection pressure provided by the introduction, persistent use and subsequent evolution of resistance to acetolactate synthase inhibiting (ALS) herbicides (Owen, 2001 cited in Owen & Zelaya, 2050).

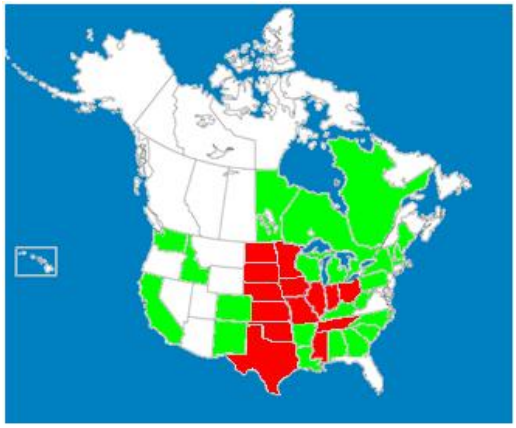


Figure 1.2: *A. tuberculatus* prevalence in North America and Canada. States and provinces containing *A. tuberculatus* are green and whereas areas containing glyphosate-resistant *A. tuberculatus* are red (1.3.2). Although *A. tuberculatus* may be found throughout North America it is principally a problematic agricultural weed in Midwestern USA. Source of data: (Heap, 2013) www.weedscience.org.

1.2.2.4 Fecundity

A. tuberculatus has a high fecundity with the potential to produce up to 2,000,000 seeds plant^{-1} (Battles *et al.*, 1998) this is an enormous quantity of seeds. Particularly when one compares the seed production figures to that of *A. palmeri* which has the potential to produce just 600,000 seeds plant^{-1} (Keeley *et al.*, 1987). This level of seed production results in a high density of *A. tuberculatus* plants and thus a competitive advantage of *A. tuberculatus* over other weeds species and crops (Horak & Loughin, 2000; Steckel & Sprague, 2004).

To summarise, the characteristics of a problematic weed include ubiquity, high seed production, delayed germination and rapid growth leading to strong competitiveness. All of these traits are present within *A. tuberculatus.* When these attributes are coupled with the tendency for the evolution of multiple herbicide resistances, it is easy to comprehend why *A. tuberculatus* may be described as "the perfect weed" (Hartzler, 2003 cited in Uscanga- Mortera *et al*., 2007.

1.3 Herbicide resistance

A rigorous definition of resistance is utilised to determine when a weed population has become resistant to a particular herbicide. Heap outlines the criteria for defining an herbicide resistant weed population:

"*The evolved capacity of a previously herbicide-susceptible weed population to withstand an herbicide and complete its life cycle when the herbicide is used at its normal rate in an agricultural situation*" (Source:Heap & LeBaron, 2001).

Heap then goes on to qualify that for a weed population to be classified as resistant, it must be present under field conditions and resistance must be confirmed through a dose response experiment. Moreover, the weed population must be problematic for the farmer and finally the resistance must be heritable (Heap, 2005).

Unconscious selection for agricultural weeds has occurred throughout the history of agriculture. Recently, farmers have selected for the contemporary weed adaptation: herbicide resistance (Heap, 2013). Herbicide resistance rapidly evolves in situations where a single herbicide is relied upon for weed control or, alternatively, where different herbicides are used but they are chosen from within the same herbicidal mode-of-action and as such induce selection pressure on the same target-site (Heap, 2006).

Herbicide resistance may arise within a population through the formation of a spontaneous novel resistant mutation (Jasieniuk *et al.*, 1996) and selection for pre-existing resistance alleles. If a novel beneficial herbicide resistant mutation arises and is propagated through repeated application of the herbicide, natural selection will act to increase the frequency of the resistance allele within the population and thus produce an herbicide resistant population. Indeed, the mutation-selection balance describes how mutations arise sporadically and thus may exist at a low frequency within a population, even in the presence of a fitness cost (Orr & Betancourt, 2001). A change from the original environment must occur to provide a positive selection pressure for a situational beneficial allele. Thus selection can act upon standing genetic variation found within a population and as such select for existing herbicide resistance alleles over generations to produce a resistant population (Vencill *et al.*,

2012). Similarly, low-dose herbicide applications may produce creeping resistance through the selection of multiple, pre-existing low-effect resistance alleles which are combined into a high level of resistance (Neve & Powles, 2005; Busi *et al.*, 2008).

Herbicide resistance has the potential to arise within a population without novel mutation or pre-existing resistance alleles. For example, the activation of mobile genetic elements such as transposons, are able to duplicate target-site genes to produce a high level of the target enzyme, capable of resisting field rate application (Gaines *et al.*, 2010). Alternatively, the migration of a resistance allele may occur between population due to pollen or seed transmission from a neighbouring resistant weed population (Vencill *et al.*, 2012).

Finally, it has been proposed that herbicide resistance may arise through the transmission of herbicide resistance alleles from transgenic crops to related wild species through pollen gene flow. The potential for resistance to evolve through introgression with transgenic crops has been documented between glufosinate resistant *Oryza sativa* (rice) and the weed sub-species of *Oryza sativa* (red rice). Where a low rate of hybridisation <1% is observed in the field resulting in the transmission of the transgenic *bar* resistant gene (Zhang *et al.*, 2003). However, transgenic gene transmission to a weed species is rare compared to the evolution of resistant weeds (Heap, 2013). Additionally, it is more common for glyphosate tolerant traits to transfer into non-GM cultivars of the same crop species than a related weed species (Mallory-Smith & Zapiola, 2008).

The evolution of herbicide resistance has occurred in 21 out of 25 herbicidal modes-of-action, within 61 separate countries, in 218 different weed species (Heap, 2013).

This number has risen abruptly since the first reported case of resistance to triazine herbicides in 1970 in a population of *Senecio vulgaris* L. (Ryan, 1970 cited in Warwick & Black, 1994)(figure 1.3).

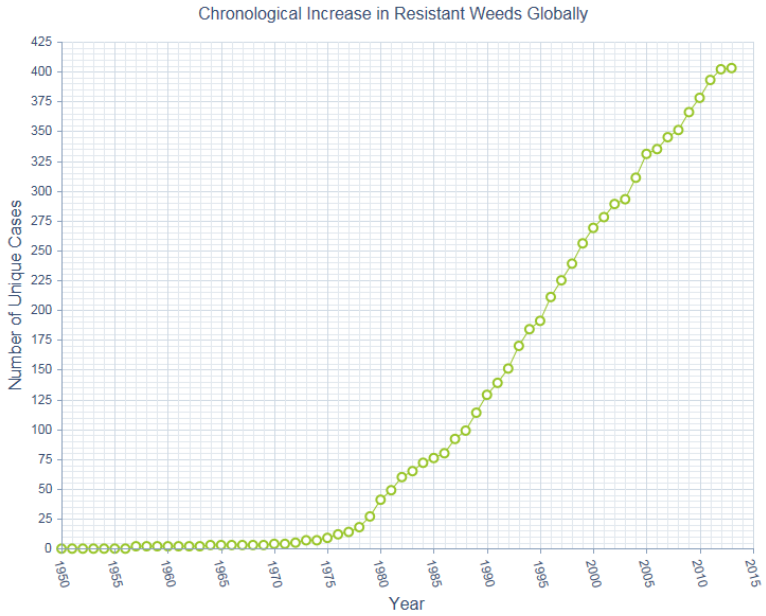


Figure 1.3: The frequency of herbicide resistant unique cases which have evolved over time. A unique case is a resistance incidence with a unique herbicide/ weed species/ mechanism of resistance. Source: (Heap, 2013) www.weedscience.com

1.3.1 Herbicide resistance mechanisms

The most common types of resistance mechanism may be classified as target-site, impaired uptake, sequestering or herbicide metabolism based. Other possible resistance mechanisms include morphological, phenological and physiological changes in the weed, however, these mechanisms are rarely found to have a major impact upon resistance (Moss, 2002). Target-site resistance may have been caused

by a sequence mutation/deletion, gene amplification or overexpression. A spontaneous target-site mutation may result from a single nucleotide polymorphism (SNP) which leads to a subtle alteration of the amino acid sequence and therefore an alteration in the shape of the target enzymes herbicide binding site, endowing herbicide resistance. Alongside herbicide resistant proprieties the resistant SNP must not significantly prevent the enzyme substrate(s) binding, thus allowing the maintenance of enzymes function. The majority of triazine, ACCase inhibitors, ALS inhibitors and dinitroaniline herbicide resistant cases are due to alterations in the target-site (Moss, 2002). For example, there are eight loci where single amino acid substitutions in the ALS enzyme can convey ALS inhibiting herbicide (Yu, 2013) and seven codon positions in the ACCase enzyme that are involved in resistance to ACCase herbicides (Kaundun, 2013).

Resistance may also result from target-site gene amplification where multiple copies of the target gene are replicated in the plant genome. For example, cultured *Nicotiana tabacum* (tobacco) plants cells have evolved resistance to sulfonylurea (an ALS inhibiting herbicide) under artificial selection due to gene amplification. Resistant plant cells were observed with 20 additional gene copies of the SuRB ARAS gene in resistant plants compared to 4 gene copies in wild type cells (Harms *et al.*, 1992). Over-expression produces increased numbers of the target gene transcript and therefore an increase in the target enzyme in a similar mechanism to gene amplification. For example, *N. tabacum* plants have been genetically transformed to over express the protoporphyrinogen oxidase target enzyme resulting in acifluorfen resistance (Lermontova & Grimm, 2000).

Metabolically resistant weed populations contain phenotypes which have evolved a superior ability to metabolise herbicides. Herbicide metabolism has often been attributed to cytochrome P450 mono-oxygenases and glutathione-S-transferase enzymes. Such metabolism mechanisms have evolved for PSII inhibitors, ALS inhibitors, ACCase inhibitors and dinitroaniline herbicidal modes-of-action (Tardif & Powles, 1999; Powles & Yu, 2010). Indeed, all four herbicidal modes-of-action are metabolised in distinct populations of *L. rigidum*. Herbicide metabolism is found in 12 species (Powles & Yu, 2010). Finally, the non-target-site mechanisms resulting in the impaired herbicide translocation from the site of application to the site of toxicity can result in herbicide resistance 4.1.3 (Feng *et al.*, 2004).

1.3.2 Resistance found in Amaranthus tuberculatus

A. tuberculatus has the propensity to evolve resistance to multiple herbicides (Mueller *et al.*, 2005). To date, resistance has been found to 6 modes-of-action in *Amaranthus tuberculatus*: Photosystem II (PS II) inhibitor herbicides (Anderson *et al.*, 1996b), acetolactate synthase inhibitors (ALS) (Sprague *et al.*, 1997), protoporphyrinogen oxidase (PPO) inhibiting herbicides (Patzoldt *et al.*, 2005), glycine herbicides (such as glyphosate) (Legleiter & Bradley, 2008), 4-Hydroxyphenylpyruvate dioxygenase (HPPD) inhibitors (Hausman *et al.*, 2011) and finally, synthetic auxins (Heap, 2013). There have been multiple reports of *A. tuberculatus* populations which have evolved resistance to three separate herbicidal modes-of-action (Mueller *et al.*, 2005; Patzoldt *et al.*, 2005; Legleiter & Bradley, 2008; Tranel *et al.*, 2011). In fact, individuals within an Illinois population of *A. tuberculatus* have been discovered containing multiple resistances through

independent resistance mechanisms providing resistance to the lethal dose application of 9 different herbicides. These herbicides span 3 different modes-of-action: PPO, ALS and PS II inhibitors (Patzoldt *et al.*, 2005). This history of herbicide resistant incidences indicate that there are ever reducing options for the control of *A. tuberculatus* using post emergence herbicide applications (Patzoldt *et al.*, 2005). Regrettably, the persistent use of glyphosate occurs within maize, cotton and soybean fields after the evolution and establishment of glyphosate resistant weeds. This is because glyphosate, a broad spectrum weed killer, will remain an effective control agent on multiple alternative weed species (Foresman & Glasgow, 2008). This continued selection for glyphosate resistance traits compromises the potential to eradicate glyphosate resistant populations. One may assume that persistent glyphosate application will lead to a continued selection pressure for a higher frequency of resistant individuals and therefore exacerbate the resistance epidemic. Indeed, since the first report of glyphosate resistant *A. tuberculatus* in 2008 (Legleiter & Bradley, 2008), populations have established in 14 USA states (Heap, 2013).

The cost of controlling a glyphosate resistant *A. tuberculatus* population in a *Z. mays* - *G. max* rotation has been estimated at \$44.25 ha year^{-1} (Mueller *et al.*, 2005). This research hoped to prompt a proactive rather than a reactive strategy to the threat of resistance (Mueller *et al.*, 2005). However, glyphosate control issues in *A. tuberculatus* were reported in Badger and Everly; Iowa as early as 1998, implying that glyphosate resistance evolution was probable and that the proactive action in 2005 is overdue (Zelaya & Owen, 2000; Zelaya & Owen, 2002; Owen & Zelaya, 2005). In 2001, Common Waterhemp populations were found to have highly

variable percentage survival responses to glyphosate application at low levels: 210 g ai ha^{-1}, a quarter of field rate applications (Patzoldt *et al.*, 2002). Subsequently, a low level of resistance was present within field populations in 2006 when the variable glyphosate response of *A. tuberculatus* was confirmed in populations from the Midwest United States with a lethal dose 90 of 1000 g ha^{-1} of glyphosate compared to the standard field rate application of 840 g ha^{-1} (Smith & Hallett, 2006). Inevitably, the first case of confirmed glyphosate resistance in *A. tuberculatus* was reported in 2008 in Missouri alongside multiple resistances for PPO and ALS inhibiting herbicides (Legleiter & Bradley, 2008).

To date glyphosate resistant *A. tuberculatus* has been reported in Minnesota, Missouri, Illinois, Ohio, Kansas, Indiana, Iowa, North Dakota, South Dakota, Oklahoma, Tennessee, Nebraska, Mississippi and Texas (Light *et al.*, 2011; Heap, 2013)(figure 1.2). Furthermore, a comparatively large area of American crop production land (~4%) was infested with herbicide resistant *A. tuberculatus*, this level of infestation is striking when compared to the levels associated with other *Amaranthus* species (figure 4; Tatnell *et al.*, 2007). On considering the extent of the resistant *A. tuberculatus* epidemic, it is clear that more research into an effective control is required.

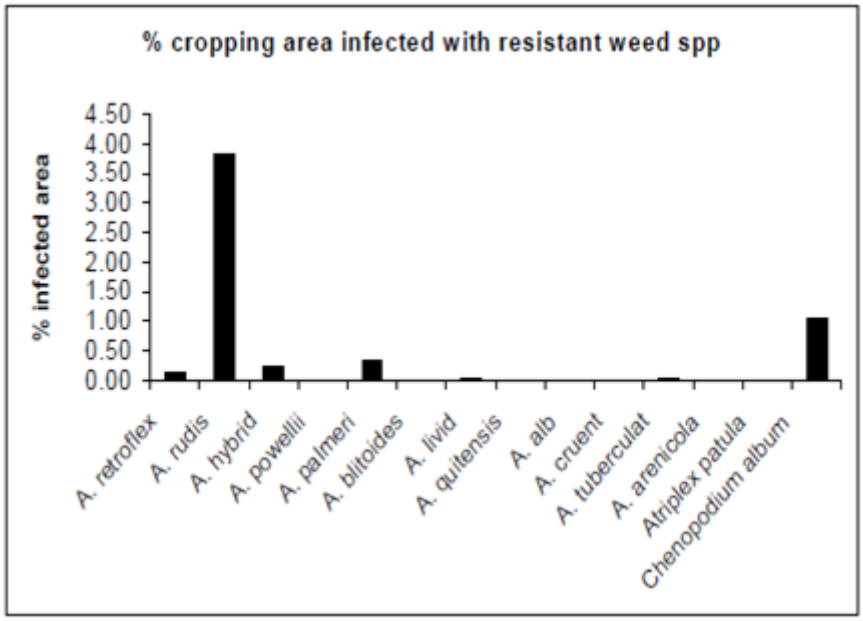


Figure 1.4: The percentage of cropped area infested with resistant weed species in the USA. Source Broad leaf weeds resistance review by Defra & PSD (Tatnell *et al.*, 2007)

1.3.2.1 The evolution of herbicide resistance in Amaranthus tuberculatus

Several characteristics of *A. tuberculatus* explain why the species has a greater tendency for the evolution of herbicide resistance. Firstly, the increased rate of herbicide resistance evolution may be due to the large population sizes and high fecundity of individual plants, with the production of up to 2,000,000 seeds per plant (Battles *et al.*, 1998), such a high offspring number permits high genetic diversity and rare mutations to occur within a population. Indeed, the positive association between resistance risk and initial seed bank density was established through the modeling of glyphosate resistance evolution in *A. palmeri* (Neve *et al.*, 2010). Additionally, *A. tuberculatus* are dioecious; this obligate out-crossing pollination method allows the rapid spread of beneficial mutations, such as herbicide resistance,

both within and between populations (Lee *et al.*, 2009). Finally, *Amaranthus* species have the potential to introgress genetic material between species. The transfer of herbicide-resistance genes has been reported from *Amaranthus hybridus* to *A. tuberculatus* (Trucco *et al.*, 2009), leading to the introgression of beneficial herbicide resistance alleles (ALS and PPO inhibitors) without an observable penalty (Trucco *et al.*, 2009). In a recent study an introgression level of <0.2% has been reported between *A. palmeri* and *A. tuberculatus,* alongside the reported transfer of the *EPSPS* gene amplification glyphosate resistance mechanism (Gaines *et al.*, 2011). This data confirms the potential to transfer resistance genes between *A. tuberculatus* and *A. palmeri,* however, the potential for this method of resistance transfer to occur naturally is impeded by the low fertility of the resulting hybrids (Wetzel *et al.*, 1999; Franssen *et al.*, 2001; Steinau *et al.*, 2003; Trucco *et al.*, 2007).

1.4 Glyphosate and glyphosate-resistant crops

1.4.1 Glyphosate

Glyphosate was developed by Monsanto and introduced in 1974, since when it has been used extensively and globally (Powles & Yu, 2010). Glyphosate (N-(phosphonomethyl) glycine) is an important herbicide due to its unique and desirable properties, for example it is cheap to produce and highly effective (Baylis, 2000). Glyphosate is a non-selective herbicide; it can therefore be applied as an effective method of removing a wide range of annual broad leaved, sedge and grass species in conjunction with the majority of perennial plants (Baylis, 2000).

It has been reported that glyphosate has desirable environmental characteristics being both immobilised and metabolised in the soil. Glyphosate has a half-life of nine days whereby the mineralisation of glyphosate can be observed in the evolution of $^{14}CO_2$, following the addition of ^{14}C labeled glyphosate to soil (Weaver *et al.*, 2007; Simonsen *et al.*, 2008). The resulting lack of persistence means that glyphosate has no residual activity in the soil and therefore no detrimental impact on subsequent crops and minimal ground water contamination (Duke & Powles, 2008). Nonetheless, glyphosate and AMPA (the main degradation product) has been found in 40 % and 83 % of 51 water systems after post-emergence glyphosate application in Midwestern United States, respectively (Battaglin *et al.*, 2005).

It was initially reported that glyphosate was not toxic to mammals or human health (Baylis, 2000; Williams *et al.*, 2000; Duke & Powles, 2008). However, there is evidence that glyphosate induces apoptosis and necrosis in human embryonic and placental cells, particularly when considering the impact of associated adjuvants (Benachour & Séralini, 2009). Glyphosate can also inhibit cytochrome P450 human hepatic enzymes to prevent the breakdown of alternate xenobiotics leading to hypothesised links of glyphosate to cancer and many additional diseases (Samsel & Seneff, 2013).

1.4.2 EPSPS Pathway & inhibition

Glyphosate inhibits the active site of the chloroplast enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (Schönbrunn *et al.*, 2001). EPSPS binds Shikimate-3-phosphate (S3P) and Phosphoenolpyruvate (PEP) to produce 5-enolpyruvylshikimate-3-phosphate. Glyphosate is a competitive inhibitor

with respect to the PEP binding site and a non- competitive inhibitor with respect to the shikimic-3-phosphate binding site (Boocock & Coggins, 1983; Schönbrunn *et al.*, 2001). EPSPS is one of the enzymes in the shikimate pathway. Inhibition from glyphosate leads to an accumulation of shikimic acid, a large proportion (30 %) of assimilated carbon is allocated to the shikimic acid pathway (Singh *et al.*, 1991), the products of which are used in the biosynthesis of aromatic amino acids, auxins and quinones (Franz *et al.*, 1997). Glyphosate inhibits this production. Several physiological responses result either directly or indirectly from glyphosate application; these include chlorophyll degradation and inhibition of auxin transportation (Cole, 1985; Baylis, 2000). The phytotoxicity symptoms of glyphosate application include chlorosis, stunting, reduction in apical dominance and ultimately plant death (Baylis, 2000).

1.5 Genetically modified crop production regimes

The creation of genetically modified, glyphosate tolerant crops (GTC) transformed agriculture within North America because it has allowed the exclusive use of glyphosate, a relatively low environmental impact herbicide, as a method of controlling weeds (Ghersa *et al.*, 2000a). Glyphosate can be applied post emergence to GTC without inflicting damage on the crop whilst allowing the removal of competitive weeds. The majority of GTC contain the *CP4* agrobacterium gene which has an analogous function to the *EPSPS* plant gene, however, it also has high tolerance to glyphosate application (Padgette *et al.*, 1991; Darmency, 2013). In addition to the introduction of the *CP4 EPSPS* glyphosate intolerant enzyme, glyphosate tolerance of GTC has been increased through the transgenic addition of

glyphosate metabolising bacterial genes such as glyphosate oxidase reductase (GOX), into crop genomes which metabolise glyphosate into aminomethylphosphonic acid (AMPA) (Zhou *et al.*, 1995; Saroha *et al.*, 1998).

Glyphosate resistant *G. max* and *Brassica napus* (canola) were introduced in 1996, *Gossypium hirsutum* (cotton) in 1997 and *Z. mays* in 1999 (Owen & Zelaya, 2005). The extent of the utilisation of GTC can be appreciated when it is considered that GTC are grown on 170.3 million hectares throughout 28 countries (James, 2012). 96% of the 30.3 million *G. max* hectares grown in North America are glyphosate resistant varieties (Dill *et al.*, 2008). GTC production systems can provide a more effective form of weed management. Indeed, the control of *Oryza punctata* (red rice) using a herbicide tolerant *Oryza sativa* (rice) over a conventional variety produced a \$50 hectare^{-1} increase in profit margins compared to a conventional crop production system (Annou *et al.*, 2001). Moreover, the weed management implementation costs associated with tillage and alternate herbicide application in a conventional system are removed through the adoption of GTC. In fact, the deviation away from conventional crop production systems has saved USA farmers a total of \$1.2 billon (up to 2005) (Gianessi, 2005).

1.6 Glyphosate resistance

1.6.1 Glyphosate resistance evolution

No cases of glyphosate resistant weed populations had been reported up until and including 1994, 20 years after the introduction of glyphosate (Dyer, 1994 cited in Powles & Yu, 2010). Additionally, in attempting to create glyphosate resistant crops,

Monsanto could not artificially induce a mutation which conferred glyphosate resistance whilst maintaining functionality of EPSPS (Bradshaw *et al.*, 1997). The delayed evolution of glyphosate resistance and the difficulties associated with inducing resistance led some scientists to believe that glyphosate was "resistant proof"; this was disproved 22 years after glyphosate introduction when the first case of glyphosate resistance was reported (Powles *et al.*, 1998). The first incidence of a glyphosate resistant weed population was reported in Australia, in *Lolium rigidum* in 1996 (Powles *et al.*, 1998). It is believed that the standardized low level application rates of glyphosate and the intensive glyphosate application which is practiced in Australia has led to the rapid evolution of resistance (Powles *et al.*, 1998; Neve & Powles, 2005). The glyphosate resistance epidemic has spread: to date there are at least 150 incidences of glyphosate resistance within 22 weed species in 19 countries (Heap, 2013) .

Unsurprisingly, a dramatic increase in glyphosate use is associated with the adoption of GM crop regimes. Therefore, GTC have increased the selection pressure for the evolution of glyphosate resistance and such regimes are indeed associated with high levels of glyphosate resistant weeds (Powles, 2008; Duke & Powles, 2008; Powles & Yu, 2010). Indeed, the introduction of GTC resulted in simplified weed management strategies. This has in turn led to a shift in the weed community from annual grasses and broad leaved perennials to broad leaved annual species that have evolved resistance to glyphosate (Johnson *et al.*, 2009). The correlation between the areas of the world in which genetically modified crops have been adopted (in 2003), and the level of glyphosate resistance which has evolved by 2010 is provided in figure 1.5 a & b. The strong association between the evolution of glyphosate resistance and

uptake of genetically modified crops can be understood when considering that GTC composed 95 % of the total genetically modified crop harvest in 2008 (Powles, 2008). In 2002, a survey was undertaken over 10% of USA crop production land and it was found that the average number of herbicidal modes-of-action applied to *G. max* crop fields had dropped from 11 in 1995 to just one (glyphosate) in 2002 (Young, 2006). It is this heavy reliance upon glyphosate which has led to a strong selection pressure for glyphosate resistant weeds to evolve (Owen & Zelaya, 2005). In reaction to the resistance problem 50% of GR maize and 25% of soybean hectares are now sprayed with an alternative mode-of-action herbicide (Dill *et al.*, 2008). The absence of tillage within glyphosate resistant crop systems has also contributed to the evolution of resistance. Tillage permits weed control through the burial of pre-existing weeds, therefore, a no-tillage regime reduces weed control diversity and thus increases the pressure on herbicides for weed control. The more diverse the control strategy the lower the selection pressure for the evolution of herbicide resistance (Neve *et al.*, 2003; Powles & Preston, 2006).

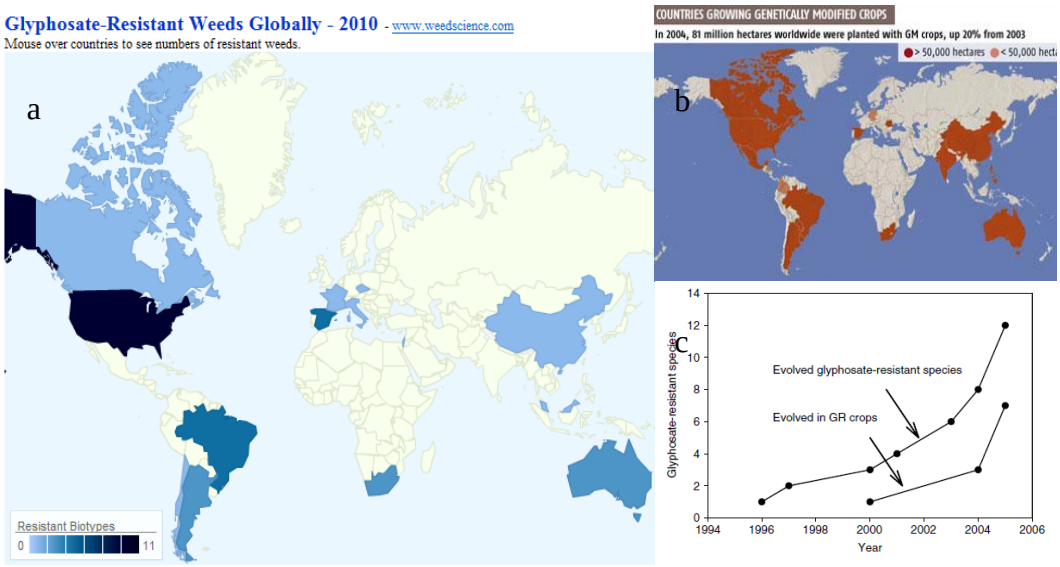


Figure 1.5: a. Genetically modified crops grown globally –source: (James, 2012).**b. Glyphosate resistant weeds that have evolved globally -2010** source: (Heap, 2013). **c.** The number of glyphosate resistant weed species that have evolve resistance in total and the number of weed species that have evolved resistance in glyphosate tolerant crop regimes (Source: Duke & Powles, 2008; Heap, 2013).

1.6.2 Glyphosate resistance mechanisms

Many target-site and non-target-site mechanisms of glyphosate resistance have evolved within weed populations. Target-site glyphosate resistance can be mediated by an alteration in the *EPSPS* target-site sequence (for example: Baerson *et al.*, 2002; Kaundun *et al.*, 2011; Jalaludin *et al.*, 2013) or gene amplification of the *EPSPS* target gene (Gaines *et al.*, 2010). A non-target-site mechanism may endow glyphosate resistance resulting in reduced translocation (for example: Lorraine-Colwill *et al.*, 2002; Koger & Reddy, 2005), such a mechanism can result from increased vacuole sequestration (Ge *et al.*, 2010). Glyphosate metabolism is not

considered to be a major mechanism of glyphosate resistance in weed populations (Powles & Yu, 2010; Duke, 2011), however, glyphosate metabolism has been reported in a population of *Digitaria insularis* (Sourgrass), alongside three additional glyphosate resistance mechanisms (de Carvalho *et al.*, 2012). A combination of glyphosate resistance mechanisms may occur within a single population, for example mechanisms resulting in impaired glyphosate translocation and *EPSPS* target-site mechanisms have been found in a *L. rigidum* population from South Africa (Yu *et al.*, 2007). For a detailed summary of glyphosate resistance mechanisms refer to 4.1.

1.7 Costs of resistance

1.7.1 The theory of costs of adaptation

Differences in fitness are required for natural selection to take place and for the process of evolution to occur (Darwin, 1859). An alleles fitness is defined as the proportion of individuals containing the allele in a population after successive generations (Primack & Kang, 1989; Lawrence, 2005; Vila-Aiub *et al.*, 2009a). The theory of costs adaptation is applied in many research areas including evolutionary biology, population genetics, plant physiology and more specifically, xenobiotic resistance (Coustau *et al.*, 2000; Vila-Aiub *et al.*, 2011). The theory of costs adaptation states that an adaptation, which confers a benefit to a novel stress, must result in a large modification of the initial phenotype and therefore, the pleiotropic impact of the adaptive allele(s) may produce a fitness cost in the original environment (Herms & Mattson, 1992; Bergelson & Purrington, 1996; Coustau *et al.* 2000; Fry, 2003; Vila-Aiub *et al*., 2011). The antagonistic pleiotropy associated with

an adapted allele may directly produce a fitness cost or discrepancies in life history traits such as biomass (McCloskey & Holt, 1990), emergence (Vila-aiub *et al.*, 2005), height and flowering time (Roux *et al.*, 2005c) which may ultimately produce a fitness cost in a resource limited environment.

The best measure of fitness is fecundity; explicitly, fitness is the ability of an organism, or a population, to survive until reproduction, the number of offspring produced and also the ability of these offspring to reproduce (Heil & Baldwin, 2002; Orr, 2009). Nonetheless, growth rate and size can be an indirect measure of fitness, if plant size correlates with reproductive output (Samson & Werk, 1986). The prediction of herbicide resistance evolution to a pesticide is influenced by any negative trade-off between the resistance trait and fitness in the absence of the selection pressure (Coustau *et al.*, 2000).

The maintenance of a resistant: susceptible polymorphism within a population under evolutionary equilibrium, can be explained by the presence of a resistance cost (Parker, 1992; Coustau *et al.*, 2000). Indeed, the fixation of a resistant mutation which is associated with a fitness cost will result in genetic load. Genetic load has occurred when a population containing a single variant of an allele has a lower fitness than the original polymorphic population (Orr & Betancourt, 2001). If there is no cost associated with an adaptation then, under recurrent selection, the adapted genotype will be selected to predominate within a population.

Fitness costs are well documented within the research area of antibiotic resistance: multiple studies have found that bacterial populations containing plasmids which endow antibiotic resistance have a slower growth rate when compared to wild type

bacteria (Lee & Edlin, 1985; Nguyen *et al.*, 1989; Bentley *et al.*, 1990; Purrington & Bergelson, 1997). Additionally, fitness costs can be associated with many adaptations within plant ecology and are not solely restricted to herbicide resistance. For example, a dramatic growth penalty was present in *Arabidopsis thaliana* (thale cress) after genetic manipulation to produce constitutively expressed salicylic acid. The production of salicylic acid conveys resistance to the pathogen *Peronospora parasitica*, however, this constituent expression is associated with drastically reduced plant size (Mauch *et al.*, 2001; Heil & Baldwin, 2002). Similarly, fitness costs can be associated with herbivory protection. A high trichome density is believed to reduce the level of herbivory experienced by *A. thaliana*. Yet in the absence of herbivores a high trichome density is found to be negatively correlated with fruit number, thus indicating that a penalty is associated with resisting herbivory (Mauricio, 1998).

1.7.2 Costs of herbicide resistance

Bergelson & Purrington (1996) conducted a survey to determine the likelihood of finding a fitness cost associated with resistance in 88 fitness cost studies which observed herbicide, disease and herbivore resistant plants. Only studies which had controlled the background genetic variation, between comparisons, were included in the analysis. Overall, 50% of the comparisons revealed the presence of a fitness cost. Furthermore, 62% of the studies on herbicide resistance revealed a fitness cost. It is hypothesized that this comparatively high cost-incidence, is due to the stronger selection pressure imposed by herbicides. However, this may also be due to the high costs associated with triazine resistance and the large number of studies investigating

this phenomenon (Warwick & Black, 1994; Bergelson & Purrington, 1996). A subsequent review, which looks specifically at herbicide resistance, emphasized the common methodological flaws of fitness cost studies, including the use of inappropriate measures of fitness, but more notably it acknowledges that fitness costs are not universal and that they are determined by the nature of the mutations and resistance mechanisms that have been selected (Vila- Aiub et al., 2009).

A fitness benefit-cost balance is associated with a herbicide resistance mutation (Roux *et al.*, 2006). The resistance benefit-cost determines if a resistance allele will be selected and established within an agricultural weed population under herbicide application (Roux et al., 2006). Inversely, the resistance cost will determine if a resistance allele frequency reduces, in the absence of herbicide application. Therefore the fitness benefit-cost balance may determine the frequency of an herbicide resistance allele in an agricultural environment under different management strategies (Roux et al., 2006). The costs of resistance can explain the maintenance of herbicide resistant (R) and susceptible (S) polymorphisms found in numerous resistant populations under continuous selection (Purrington, 2000).

1.7.3 Costs associated with triazine resistance

As previously mentioned there is a wealth of studies investigating the occurrence of fitness costs associated with triazine resistance. Triazine herbicides target the plastoquinone binding site of the D1 protein PSII subunit, thus preventing the PSII electron transport system and inhibiting photosynthesis. A single resistance endowing mutation (Ser-264–Gly) has evolved globally and dominated reports of triazine resistance. Twenty-five studies have reported a fitness cost associated with

the Ser-264 mutations (Warwick & Black, 1994). The D1 protein has been highly conserved throughout evolutionary history due to the essential function of the protein. Therefore, it is no revelation that lower photosynthetic production is associated with a mutated enzyme and this less efficient D1 protein directly translates into a fitness cost.

1.7.4 Absence of fitness costs

The ACCase1781 resistant mutation was introgressed from the *Setaria viridis* into *Setaria italica* individuals, the resulting progeny experienced 7 rounds of backcrossing to *Setaria italica* individuals and homozygous (RR & SS) individuals were selected for use in the experiment. Faster growth and increased grain number was observed in *Setaria italica* individuals containing the ACCase Ile- 1781- Leu resistant mutation (RR) when compared to susceptible plants (SS) (Wang, 2010). However, when experimental trials were undertaken in the field, resistant and susceptible phenotypes were seen to have equal fitness. Wang (2010) hypothesises that the absence of a fitness cost may be due to a linked beneficial gene associated with the resistance allele. The lack of a fitness cost means that a resistance allele is unlikely to be associated with a frequency reduction of the resistance allele in the absence of selection.

1.7.5 Compensatory adaptations

Under environmental conditions of persistent selection, fitness costs may be ameliorated overtime either through the substitution of the resistant mutation, to a mutation not associated with a fitness cost (Cohan *et al.*, 1994) or through

compensatory adaptations (Fisher, 1928 cited in Paris *et al*., 2008; Vila-Auib *et al*., 2011). The compensatory mutations may be a novel mutation arising within the population (Maisnier-Patin & Andersson, 2004) or a pre-existing allele within the background genetic diversity of the population (Paris *et al.*, 2008). Paris et al.,(2008) looked specifically at herbicide resistance and found that the background genetic diversity can alter the level or presence of a fitness cost associated with a resistance allele. Therefore, a resistant population under persistent pressure will select for traits which improve fitness over time. Moreover, experiments using the model organism *Pseudomonas fluorescens* found that distinct colonies would evolve to survive the air-broth interface of a microcosm; however, the evolved individuals experienced a fitness penalty in the original growth environment. Over time, the penalty was found to diminish and this is believed to be due to compensatory adaptations (MacLean *et al.*, 2004). These findings suggest that any management strategy implemented to increase a fitness costs, must be implemented to produce a reduction in the frequency of a resistance allele before the amelioration of costs within resistant populations.

1.7.6 Mechanisms of fitness costs

The resistance mechanism can influence the anticipated fitness cost mechanism. The resource allocation theory states a resistant individual will divert resources away from growth and reproduction and allocate increased energy resources to defense, resulting in a cost of adaptation (Bergelson & Purrington, 1996). Similarly, the growth- differentiation balance (GDB) describes the trade-off between the adaptive benefit in the presence of the stressors and any potential adaptive cost in the absence of the stressors (Herms & Mattson, 1992). Specifically, this theory describes a

resistance cost, which results from the resource diversion away from carbon fixation and nutrient acquisition to plant herbivore defence. However, constituent expression of multiple target-site gene copies, such as that observed in glyphosate resistant *A. palmeri* may cause an energetic drain on resources to produce an energetic cost analogous to the defense resource allocation outlined above (Gaines *et al.*, 2010; Powles & Yu, 2010). By contrast, a target-site mutation requires no alteration in resource allocation; however, the mutation may impact upon the efficacy of enzyme function (Vila-Aiub *et al.*, 2005; Tardif *et al.*, 2006; Menchari *et al.*, 2007). Alternately morphological resistance is likely to result in structural growth limitations, allocation costs and self-shading whereas temporal escape mediated resistance may result in a restricted growth period (Purrington, 2000). Furthermore, herbicide resistance traits may result in biological interactions and ecological costs such as increased susceptibility to pests (Purrington, 2000; Gassmann & Futuyma, 2005).

1.7.7 Amplification of fitness costs

It is commonly believed that fitness costs can be amplified in the presence of intra and inter-specific competition for resources. The cost associated with a cytochrome P450 mediated resistant *Lolium rigidum* population was maintained under inter-specific competition with wheat (Vila-Aiub *et al.*, 2009b). Further evidence to support the presence of fitness costs under competition can be seen in the stress response to wounding induced by jasmonate application in *Nicotiana attenuata.* Jasmonate induced *Nicotiana attenuate* produced fewer seeds with lower nitrogen content when grown in competition with un-induced individuals (Van Dam &

Baldwin, 2001). Conversely, Purrington (2000) argues that many studies have not found fitness costs associated with resistance in a nutrient limited environment. Purrington (2000) then goes on to quote four studies that do not exhibit higher fitness costs in resource limited environments. It is suggested that a limitation in nutrients promotes a standard stress response in S individuals equal to that pre-exhibited in R individuals which is of a level that cannot be enhanced further.

Complex ecosystem interactions may also amplify the level of an observed fitness cost. For example, the fitness cost associated with target-site triazine resistant *Amaranthus hybridus* was increased by 360 % in the presence of the folivory beetle *Disonycha glabrata* (Gassmann & Futuyma, 2005). These results indicate that the resistant individuals had an increased susceptibility to herbivory and that future studies should be conducted under conditions that simulate the complexity of the environment in which resistance is selected.

1.7.8 The costs of glyphosate resistance

A number of experiments have found evidence of small fitness costs associated with glyphosate resistance. A negative trade-off in *Ipomoea purpurea* half sibling seed families was observed between seed production in the presence and the absence of glyphosate, under competitive field conditions (Baucom & Mauricio, 2004). The most tolerant seed lines produced 35 % fewer seeds compared to the most susceptible seed line when grown in the absence of glyphosate (Baucom & Mauricio, 2004). These findings show the potential for a fitness cost associated with glyphosate tolerance in the presence of competition. Unfortunately, the mechanism of

glyphosate tolerance was not reported, therefore the findings cannot be placed in the context of the resistance mechanism. Furthermore, glyphosate resistant *Lolium rigidum* population with a resistance mechanism resulting in impaired translocation of glyphosate, produced 7.5 % fewer seeds at low crop densities when compared to susceptible phenotypes isolated from the same population (Pedersen *et al.*, 2007). Additionally, the glyphosate resistance trait experiments which investigated *L. rigidum* populations containing a resistance mechanism resulting in reduced translocation, found a reduction in the glyphosate resistance allele frequency in the absence of herbicide application (Preston *et al.*, 2009). Further to weed studies, evidence has been found for pleiotropic costs associated with genetically modified glyphosate tolerant crop varieties (Darmency, 2013). For example, a glyphosate tolerant cultivar of soybean was found to have a 17-58 % reduction in yield grown under the presence of weed competition when compared to normal cultivars (Shaw *et al.*, 2001).

Comparison of resistant and susceptible individuals in the absence of glyphosate can reveal either: the presence of a fitness cost (Baucom & Mauricio, 2004; Wakelin & Preston, 2006; Pedersen *et al.*, 2007), no cost (Zelaya *et al.*, 2004; Wang *et al.*, 2010) or in some cases an increase in fitness (Wang *et al.*, 2013). The potential relationships which may be found in comparisons between discrete R and S populations are illustrated within figure 1.6a. A population containing a quantitative resistance trait will contain phenotypes with a unique level of resistance across a continuous spectrum. Therefore the level of resistance within an individual seed family can be correlated against fitness in the absence of glyphosate. As

demonstrated in figure 1.6a, a slope of 0 (c) indicates the lack of a fitness cost whereas a significant negative correlation (figure 1.6b) would indicate the presence of a fitness cost (Strauss & Agrawal, 1999; Baucom & Mauricio, 2004). Such a fitness trade-off was associated glyphosate resistance in *Ipomoea purpurea* (Baucom & Mauricio, 2004).

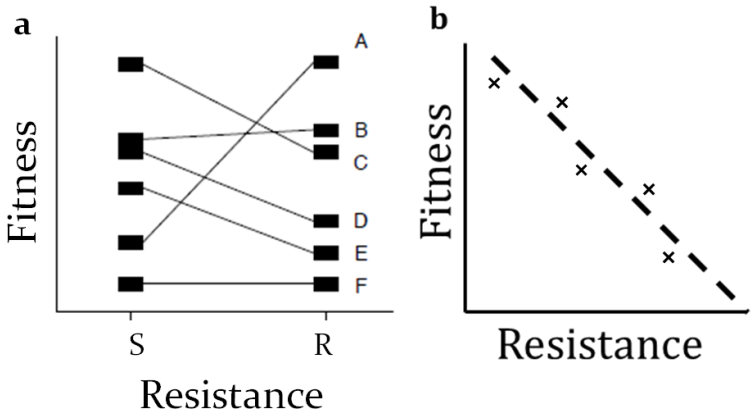


Figure 1.6: Graphical description of fitness data a. The fitness response in a classical fitness cost experiment using two phenotypes R and S, positive, negative and no relationships are depicted. A & B show a fitness advantage, C, D & E show a fitness cost and F shows no cost in the absence of the selection pressure. **b.** A negative fitness trade-off associated with resistance measured across a continuous resistance spectrum. Source: adapted from Strauss and Agrawal (1999).

Glyphosate binds directly to the active site of EPSPS, therefore it was initially proposed that the majority of glyphosate target-site mutations which result in resistance will also interrupt enzyme activity, and so produce a high fitness cost (Bradshaw *et al.*, 1997). This hypothesis is supported by the Gly-101-Ala substitution, which is a mutation artificially created in *Petunia spp.* through site-

directed mutagenesis. This mutation conveyed a high level of resistance but drastically reduced enzyme function (Padgette *et al.*, 1991). By contrast the amino acid substitutions which are commonly found within natural populations (Pro-106 to Serine, Alanine, Leucine or Threonine), narrow the active site of EPSPS to convey weak glyphosate resistance by decreasing the affinity of the enzyme for glyphosate, whilst still preserving enzyme functionality (Powles & Preston, 2006; Yu *et al.*, 2007). However, as yet, research has not determined the fitness costs associated with the P106 mutations nor indeed the *EPSPS* gene amplification mechanism (Powles & Yu, 2010; Darmency, 2013). There has, however, been documentation of a difference in a key life history trait associated with impaired translocation, whereby lower total seed number and higher individual seed weight is observed in glyphosate resistant *Lolium rigidum* (Pedersen *et al.*, 2007; Powles & Yu, 2010). Although the total resistant phenotype seed number is lower than the susceptible phenotype, the seeds are larger and therefore result in an equivalent reproductive biomass production.

1.7.8.1 Amplification of glyphosate resistance fitness costs

Glyphosate resistant *Lolium rigidum* reveals lower seed production in resistant phenotypes at low and no wheat densities (Pedersen *et al.*, 2007). This seed number reduction was not observed at high crop densities which means that the discrepancy may not translate into fitness cost within the field (Pedersen *et al.*, 2007). However, this fitness cost was later confirmed when the population was grown under the absence of glyphosate selection, revealing a reduction in the proportion of the

resistant phenotype within the population of 34% over 3 years (Preston & Wakelin, 2008).

1.7.9 The impact of resistance mechanisms on the costs of adaptation

The verification of the mechanism of resistance will support the interpretation of the fitness results because the presence or absence of costs of resistance is dependent on the mutation(s) that cause resistance. The mechanism of resistance determines both the extent and the presence of a fitness cost. Indeed, the fitness costs associated with two different resistant mutations on two genes can vary from 0 to 89 % and even two different mutations on the same resistance gene can alter the magnitude of the observed fitness penalty (Roux *et al.*, 2004). These results imply that the mechanism and mutation which endows resistance must be known before determining whether an herbicide resistant population is associated with a fitness cost. Therefore, it is important to determine the mechanism of resistance within a population because it directly informs the application of fitness results and the potential for a fitness costs within a population. An example of the influence of a specific resistant target-site mutation on associated fitness costs can be seen in ACCase resistant *Alopecurus myosuroides,* whereby a reduction in weight, height and seed production is associated with a Asp- 2078-Gly point mutation in the ACCase gene but not Ile - 1781-Leu or Gly- 2041-Asn mutations (Menchari *et al.*, 2008). Moreover, the comparison of the fitness costs associated with–ACCase resistance in *L. rigidum* show the decreased emergence of target-site resistant seed under shallow burial (1-4 cm) compared to the susceptible and ACCase metabolising phenotypes (Vila-Aiub *et al.*, 2005).

To add to the complication of fitness cost interpretation, the strength of a fitness cost is strongly dependent upon the genetic background of the population and individual (Paris *et al.*, 2008; Vila-Aiub *et al.*, 2009a). Therefore, ideally, multiple resistant populations containing the same mechanism of resistance should be studied to determine the potential application of fitness penalty results.

1.8 Integrated weed management

Integrated weed management (IWM) aims to maintain yields in a sustainable fashion whilst minimising agricultural dependency on chemicals through incorporating non-herbicidal weed control measures (Naylor & Drummond, 2002; Vencill *et al.*, 2012). IWM also aims to achieve an economically acceptable level of weed control whilst protecting and enhancing the environment (Naylor & Drummond, 2002). Indeed, Mas et al., (2010) found that the manipulation of the crop production system, in terms of the preceding crop and the presence of tillage and to a lesser extent soil productivity, all acted as a selection pressure for different weed functional traits. Therefore the alteration of a management strategy can impact upon the weed species community. Specifically, IWM strives to prevent herbicide resistance weed populations from evolving through multiple strategies. Resistant management strategies include using different herbicidal modes-of-action through sequential applications, mixtures and rotations (Vencill *et al.*, 2012). Non-herbicide weed management strategies used to prevent the establishment of herbicide resistant populations can be cultural such as sowing date and crop rotation or mechanical involving multiple tillage practices (Beckie, 2006; Vencill *et al.*, 2012). Preventative measures may be implemented as a reactive management strategy to prevent the

migration of resistant weeds between fields by, for example, the cleaning of agricultural equipment to remove weed seed (Legere *et al.*, 2000; Naylor & Drummond, 2002; Vencill *et al.*, 2012). These strategies are a proactive approach to the management of herbicide resistance and may help the prevention of resistance evolution and migration. The impact of different management techniques for resistance prevention and management may be obtained through the use of models.

1.8.1 Using fitness costs in management strategies

1.8.1.1 Modelling herbicide resistant evolution

Mathematical modelling can be used to predict the evolution and spread of herbicide resistance; it can also allow the tailoring of management practices to mitigate the risk of an herbicide resistance epidemic (Vila-Aiub *et al.*, 2005). Neve et al. (2010) modelled the impact of different management strategies on the evolution of glyphosate resistance in *Amaranthus palmeri* (Neve *et al.*, 2010). The model can be used to predict the resistant genotype frequency within a population over time and looks specifically at GTC fields. The model simulates the effect of altering the number of annual glyphosate applications. The results indicated that an additional early application of a residual herbicide, effectively reduced the risk of resistance. The adoption of best possible management strategies in areas, which have not yet become infested with glyphosate resistant *A. palmeri* can mitigate the risk of the glyphosate resistance evolution (Neve *et al.*, 2010). Therefore, models are a valuable approach in the arms race against resistant weeds.

The determination of discrepancies in fitness, between resistant and susceptible genotypes can be used to establish the dynamics of resistance evolution. Models such as the one developed by Neve et al., (2010) do not currently account for any potential fitness costs associated with resistance. However, the addition of such parameters should enhance the accuracy of the model predictions. Furthermore, it is important to consider the impact of an individual's genotype on the dominance profile of the alleles and how this will impact on the resulting fitness of an individual when constructing a model. Where resistance is controlled by a monogenic trait, the impact of dominance can be distinguished to aid the model resistance allele frequency dynamics. Moreover, the dominance profile of the pleiotropic traits associated with resistance allele may vary depending on the resistance allele. For example, a dominant fitness cost associated with a monogenic resistance trait will be exhibited in individuals containing both homozygous resistant (RR) and heterozygous (RS) alleles. Indeed, nine *Arabidopsis thaliana* lines with separate mutations to ALS, cellulose synthesis inhibitors or synthetic auxin herbicides were associated with recessive, dominant or under-dominant fitness costs and the fitness dominance profiles did not relate to the dominance profile of the resistance trait (Roux, 2004). For example, the simulation of a dominant herbicide resistance trait with an associated dominant fitness cost in an out-crossing species will change in frequency, dependent upon the management strategy adopted (Roux *et al.*, 2008).

Specifically, the most effective strategy that resulted in a reduction of the resistance trait was a herbicide treatment pattern with two herbicidal modes-of-action, in a moderate level of temporal (rotation between two herbicides) or spatial (alternate herbicides applied in strips across a field) heterogeneity (Roux *et al.*, 2008).

Polygenic herbicide resistance mechanisms are neither well characterised nor well accounted for within resistant management models. A population containing polygenic resistance will contain multiple mechanistic resistance alleles producing a quantitative resistance trait. Each of the resistance alleles contributing to polygenic resistance may be associated with a pleiotropic fitness cost and each allele associated with a different dominance profile. Encouragingly, Roux et al. (2005b) found that increasing numbers of resistance alleles, which endow resistance to different herbicides exhibited additive fitness costs. Such findings indicate that a multiple resistant population will be less fit in the original environment and therefore the frequency of multiple resistant individuals will decrease in the absence of selection.

1.8.1.2. Practical application of resistant fitness costs

Fitness cost information can also be used in a more direct way to predict the efficacy of management practices in reducing the level of resistant genotypic frequencies observed within the field. This can be achieved through the accentuation of fitness costs which are found to be associated with herbicide resistance (Cousens & Mortimer, 1995; Jordan, 1999). It is evident that the discrepancies in life history traits associated with herbicide resistance may be asymmetrically selected under specified management conditions leading to a reduction in resistant phenotypes. A direct example whereby a change in farming practice can lead to a reduction specifically in the R allele under the shallow burial of ACCase resistant *Lolium rigidum* (isolated from the SLR31 strain). Burial causes an alteration in the light level exposure and results in reduced germination in resistant individuals when compared to a susceptible SLR31 isolate. These findings indicate that tillage has the

potential to reduce the resistance allele through manipulation of the associated fitness costs (Vila-Aiub *et al.*, 2005).

1.9 Objectives

The following experiments aim to determine the level of glyphosate resistance within three *A. tuberculatus* populations from Minnesota. Subsequent experiments, will aim to determine the mechanism of glyphosate resistance, specifically whether resistance is caused by EPSPS target-site modification, target-site gene amplification or mechanisms resulting in reduced translocation of glyphosate. The putatively resistant populations will be segregated to create seed lines containing phenotypes with distinct resistance levels. This seed material will be used to determine if there is a fitness cost or a growth penalty associated with resistance when plants are grown in the absence of glyphosate. Final experiments will investigate the impact of competition on the life history traits associated with resistant and susceptible seed lines. Any observed resistant life history trait discrepancies associated with resistance are increased in the presence of competition to produce an associated fitness cost.

2.0 Confirmation of glyphosate resistance within three *A. tuberculatus* populations from Minnesota

2.1 Introduction

2.1.1 Crop production systems in the USA

In 2012, 69.5 million hectares of genetically-modified crops were grown throughout the USA (James, 2012). Moreover, 93 % of *Glycine max* (soybean), 80 % of *Gossypium hirsutum* (cotton) and 73 % of *Zea mays* (maize) grown within the USA contained the Roundup Ready ^{TM} glyphosate-tolerant (GT) trait (USDA, 2012). The GT trait is one of the most ubiquitous genetically modified crop traits utilised in the USA (USDA, 2012). This technology promotes crop yield enhancement through the effective control of numerous, problematic weeds. Predictably, the widespread adoption of GT crop varieties has led to the extensive utilization of glyphosate. Indeed, an estimated 67 million ha of USA land was treated with glyphosate in 2006, within the GT maize, cotton and soybean crop production systems (Foresman & Glasgow, 2008). Furthermore, 70 % of GT crop production systems in the northern states of the USA, conduct 2-3 glyphosate field applications per year (Foresman & Glasgow, 2008). This high glyphosate utilization has provided a strong selection pressure for the evolution of glyphosate resistant weeds.

2.1.2 Crop production systems within Minnesota

Minnesota is a major agricultural state and ranks fourth in the USA for total crop production profits (Ye, 2011). The predominant crops that are produced in Minnesota include maize, soybean, *Beta vulgaris* (sugar beet) and *Triticum* spp. (wheat) (Ye, 2011). The Corn Belt is partly located in south Minnesota, this region produced 2954000 ha of corn during 2010 (USDA, 2011). Therefore, maize production is of particular importance for Minnesota. As with the USA in general, GT crop technology has been implemented across Minnesota with 91 % of soybean and 69 % of maize containing the Roundup Ready ™ GT trait (USDA, 2012).

Many fields throughout Minnesota practice a conservation tillage system. Conservation tillage eliminates or significantly reduces soil disturbance, thus improving soil organic carbon quantity, soil water retention and preventing soil erosion (Kemper & Derpsch, 1981; Reeves, 1997). Antagonistically, conservation tillage promotes the persistence of small seeded annual weeds such as *Amaranthus tuberculatus* (Owen, 1997). Indeed, this practice, combined with the evolution of ALS resistance, has allowed *A. tuberculatus* to dominate Midwest USA agricultural weed communities (Horak & Loughin, 2000). It is projected that the widespread prevalence of glyphosate resistant *A. tuberculatus* and the lack of an effective alternative control method, will result in a departure from conservation tillage practices (Price *et al.*, 2011).

2.1.3 Glyphosate resistance in Minnesota

A. tuberculatus has an extensive distribution across the northern states of the USA and is a widespread problem within Minnesota (Powles, 2008). Glyphosate resistant *A. tuberculatus* populations occupy an estimated 501 – 1000 infested sites and over 4050- 40500 infested hectares, and the problem is growing (Heap, 2013). In addition to *A. tuberculatus*, two more weed species have evolved resistance to glyphosate within Minnesota. Specifically, *Ambrosia artemisiifolia* and *Ambrosia trifida*, which evolved resistance between 2006 and 2008 (Heap, 2013).

Glyphosate resistant weed evolution has followed the widespread adoption of genetically modified crop production regimes within Minnesota in the early 2000's. All incidences of glyphosate resistance, which have evolved within Minnesota, have occurred within GT soybean crop production regimes (Heap, 2013). Therefore, it is probable that the lack of diversity in control methods has rendered the GT soybean systems prone to the evolution of glyphosate resistant weed populations. The selection pressure for glyphosate resistant weeds within GT crop regimes is high. In 2006, 55% of Northern USA growers reported applying glyphosate, exclusively as the sole herbicide treatment (Foresman & Glasgow, 2008). The extent of the glyphosate resistant weed infestation problem, across the USA, is demonstrated through the fact that 24% of Northern USA growers estimated their land to contain glyphosate resistant weeds (Foresman & Glasgow, 2008).

2.1.4 Dose response analysis

Within the discipline of weed science, a dose response experiment may be conducted in order to achieve a number of objectives. These objectives include, the quantification of weed and crop herbicide susceptibility, the comparison of different herbicides, the impact of safeners and adjuvants on herbicide efficacy and finally determining the level of herbicide tolerance or resistance present within putatively resistant weed populations (Seefeldt *et al.*, 1995). A dose response analysis which is used to quantify herbicide resistance in a weed population requires the treatment of putatively resistant weeds with a series of herbicide doses; the population response is compared to that of a standard sensitive population. Subsequently, a log-logistic dose response analysis can be utilized to quantify the relative level of resistance in weed populations (Seefeldt *et al.*, 1995). Here the log-logistic dose response analysis has been used to quantify the level of glyphosate resistance within three populations of *A. tuberculatus* from Minnesota.

2.1.5 Objective

The primary objective of this chapter is to quantify the level of glyphosate resistance in *Amaranthus tuberculatus* populations from Renville, Dumont and Holloway, Minnesota, USA. This is achieved by comparing the proportional survival and growth reduction of suspected resistant populations with standard susceptible populations after glyphosate application. Ultimately this analysis will aid the identification of glyphosate resistant experimental populations for subsequent fitness cost experiments.

2.2 Methods

2.2.1 Seed material

The initial dose response experiment was conducted on one standard sensitive population and three suspected resistant populations of *A. tuberculatus*. Seed material had been collected from *A. tuberculatus* plants in agricultural fields exhibiting poor control following glyphosate application. The fields were located in Dumont (N 45.71, W 96.42), Holloway (N 45.24, W 95.91) and Renville (N 44.78, W 95.21) in Minnesota, USA. Renville seed was collected from a field containing a glyphosate-resistant variety of soybean in 2007; the field had been treated with four applications of glyphosate in the year of seed collection. Holloway and Dumont populations were collected from GT soybean fields in 2008, both fields had been treated with three applications of glyphosate in the year of seed collection. Each of the fields contained approximately 20-55 surviving *A. tuberculatus* plants m^{-2}. Collections were made from approximately 100 surviving female plants sampled across areas of poor control. The high survival of *A. tuberculatus* plants after multiple rounds of glyphosate application led to the strong suspicion that glyphosate resistance had evolved. GT crops were grown continuously within the fields for 6 to 8 years prior to the evolution of putative glyphosate resistant *A. tuberculatus.* Therefore, the *A. tuberculatus* populations will have experienced a minimum of one glyphosate application per year over this period.

The sensitive control seed material used in the Jealott's Hill dose response is termed PP1. PP1 has been previously characterized as susceptible to glyphosate. This population has not been exposed to glyphosate application and was sourced from Herbiseed, (identification number: PS-3010 PV3388). The S2 sensitive control seed material used in the Wellesbourne dose response has also been characterized as glyphosate susceptible and was sourced from Azlin Seed Service, (identification number: 2008 USA 10 PS2414 PV2753). Suspected resistant seed material has been provided by Syngenta (Location; identification number): Renville: 2009USA01 PS2567 PV2927, Holloway; 2009USA02 PS2569 PV2928, Dumont; 2009USA03 PS2569 PV2929.

2.2.2 Jealott's Hill Dose response

Renville, Dumont, Holloway and PP1 sensitive seeds were sown onto leveled seed trays filled with 50:50 compost: peat (John Innes compost No.2) and covered with vermiculite. Seed trays were placed under glasshouse conditions of 24/18 ^{o}C (day: night, 16:8 hours) at 65% relative humidity, with an approximate photon flux density of 250 μmol quanta m^{-2} s^{-1}. The porous matting beneath seed trays was watered as required. Following germination, seedlings were individually transplanted into 7.5 cm pots filled with the aforementioned soil mix, three weeks after sowing. All transplanted seedlings were irrigated as required and maintained under the glasshouse conditions described above, throughout the course of the experiment. Glyphosate was applied at the three-four leaf stage, 2 weeks after transplanting at doses of 0, 60, 120, 420, 840, 1680, and 3360 g ai ha^{-1}. The doses were selected to provide a uniform description of the dose response on a logarithmic scale (Seefeldt

et al., 1995). The field rate of glyphosate application for the USA is 840 g ai ha^{-1}. Therefore the selected doses encompass a range from one eighth to four times the field rate. Plants were treated with glyphosate using a CO_2-powered cabinet sprayer with a single Teejet flat fan spray nozzle (11002VS). A spray volume of 200 L ha^{-1} was applied at a pressure of 200 kPa, 30 cm above the soil level. Glyphosate was applied as the diammonium salt (Touchdown IQ) and the formulation contained 360 g l^{-1} of the active ingredient. The adjuvant Agral 90 was used at a concentration of 0.25% v/v for all doses.

The experimental design consisted of 36 replicate plants for each of the 28 treatments. Treatments consisted of 7 doses and 4 populations to produce a total of 1008 plants. Plants were placed in a randomized block design (36 blocks) and maintained under the aforementioned glasshouse conditions. Survival was recorded 21 days after treatment. Assessments of plant survival and mortality were performed according to set criteria in order to minimize the potential for experimenter bias. Conclusive symptoms of mortality included necrosis of apical meristems, complete necrosis of plant biomass and disintegration of the root system (such that the plant could be removed from the soil after minimal force was applied); plants that did not display conclusive symptoms of mortality were classified as alive. This experiment was conducted at Syngenta, Jealott's Hill, Bracknell.

2.2.3 Wellesbourne dose response

The dose response experiment was repeated using Dumont and Renville seed material, these two populations having been identified as the most resistant populations in the Jealott's Hill dose response experiment. An S2 sensitive population was selected as the standard sensitive population in the place of the PP1 sensitive population used in the Jealott's Hill dose response experiment. The S2 sensitive population; represented a similar morphology to the resistant populations and had been sourced from Azlin Seed Service which is an American based seed company. The S2 sensitive population acted as a sensitive control to determine the magnitude of resistance which has evolved in the two selected populations.

Seeds for the experimental plants were germinated in 9 cm Petri-dishes containing 2 layers of filter paper and 3.5 ml of de ionised H_2O. All Petri-dishes were sealed with parafilm, enclosed in foil, and incubated at 35/25^{o}C (14:10 h; Day/Night). On germination, seeds were individually transplanted into modular trays filled with fine grade sphagnum moss peat: sand; 24:1, (pH 5.5 – 6.0; N = 150, P = 200, K = 200 mg/litre; Levington growing media: FS2). Two weeks after transplanting, plugs were transferred individually into 9 cm pots containing medium grade sphagnum moss peat 100% (pH 5.5 – 6.0; N = 200, P = 150, K = 200 mg/litre; Levington growing media: M2). Glasshouse growing conditions were 25/20 ^{o}C (16:8 h; day/night) with supplementary lighting. There were 30 replicate plants within each of the 21 treatments. Treatments consisted of 7 glyphosate doses and 3 populations (Renville, Dumont and S2 sensitive). This amounted to a total of 630 experimental plants with each plant grown in an individual pot. The Dumont and Renville populations were

treated with 0, 60, 120, 420, 540, 840 and 1680 g ai ha^{-1} of glyphosate whereas the S2 sensitive population was treated with 0, 30, 60, 120, 180, 420 and 840 g ai ha^{-1}. Plants were treated at the three-four leaf stage, 2 weeks after repotting. Herbicides were applied using a Berthoud Velmorel 200 pro knapsack sprayer and a Deflector Anvil Polijet nozzle (D/1.2/1). Nozzle height was 40 cm above the ground, the walking speed was set 2 km hr^{-1} with the use of a metronome and pressure was set at 100 kPa to deliver a spray volume of 446 L ha^{-1}. Pots were placed in a randomized block design with 30 blocks each containing a single replicate from each treatment. Plants were harvested 21 days after treatment and measurements were taken of above ground fresh and dry biomass alongside the visual survival and mortality assessment (outlined in section 2.2.2). The experiment was conducted at Wellesbourne Campus, University of Warwick.

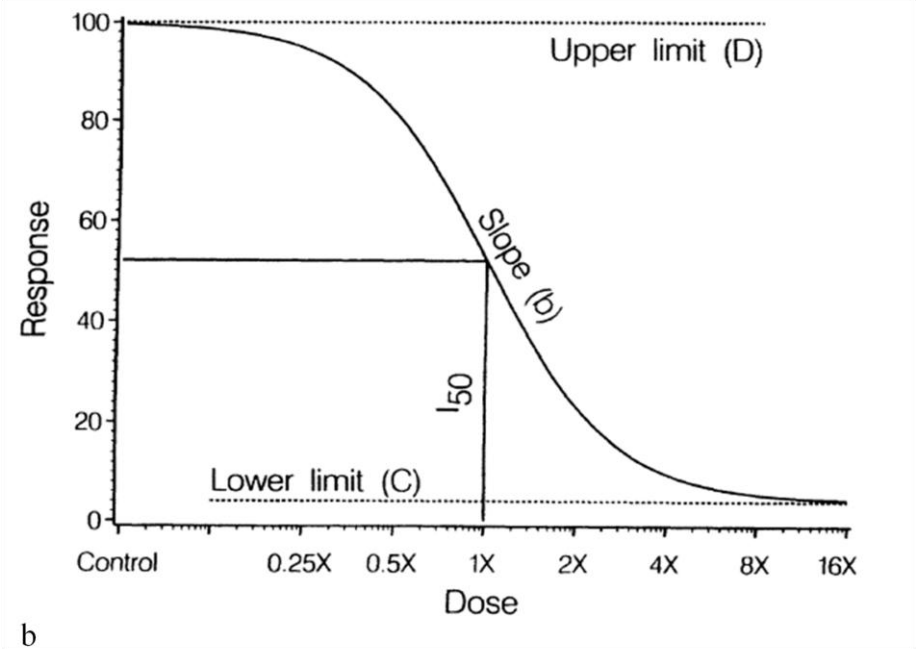
2.2.4 Data analysis

Data analysis was conducted in R (R version 2.15.1: 2012-06-22) (R Development Core Team, 2009) using the drc package version 2.1-4 (Ritz & Streibig, 2005). The three common models fitted do dose response survival and biomass data are termed the log-logistic model and the Weibull's models (1 & 2). The standard four parameter log-logistic model is depicted in figure 2.1a where the four parameters are upper asymptote, lower asymptote, slope and point of inflection. Furthermore, the Weibull's models are depicted in figure 2.2, these models account for any asymmetry in the data around the point of inflection.

2.2.4.1 Survival data

A two parameter log-logistic model (Eq. 2.1b) was fitted to survival data where the upper limit was constrained to 1 and the lower limit to 0. An upper limit of 1 indicates that all individuals survive in the control treatment and that all individuals die at the highest treatment dose. A binomial error structure specified for survival data. The resulting model can be utilised to derive descriptive values of the population dose response; for example, the lethal dose at which a given proportion of individuals are killed. A goodness-of-fit test based on the Pearson's chi-squared test determines the model fit of binomial data (Bates & Watts, 1988; Crawley, The R book p516, p549). A non-significant P- value (> 0.05) produced by the goodness-of-fit test indicates no deviation from the theoretical expected model and subsequently no lack-of-fit to the model and thus the dose response model can be used to describe the relationship between variables.

a

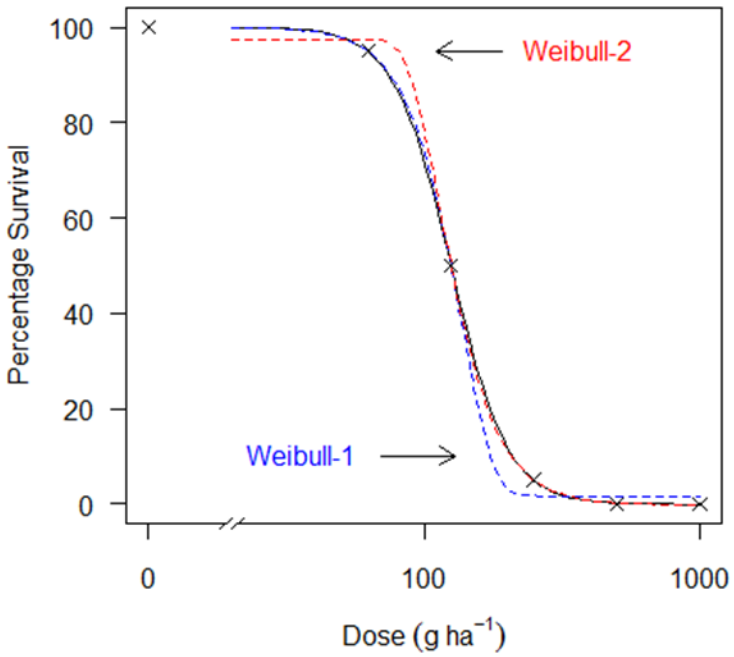


b

$$y = f(x) = C + \frac{d}{1 + \left(\frac{x}{LD_{50}}\right)^{b}} \qquad = C + \frac{d}{1 + exp\left[b\left(log(x) - log(LD_{50})\right)\right]}$$

Figure 2.1: a) An example four parameter log-logistic dose response model. (Seefeldt *et al.*, 1995) The parameter values are D = 100, C = 4, I_{50} = LD_{50}= 1X (and *b* = 2 (Seefeldt *et al.*, 1995), **b) Equation 2.1: The log-logistic two and three parameter model equation** used to describe the relationship between plant response y and herbicide dose x. The three variable parameters are: *b*- slope, *d*- upper limit and LD_{50} - lethal dose which kills 50 % of individuals. The upper limit (*d*) is set to 1 for a 2 parameter model (Seefeldt *et al.*, 1995).

a



b

$$y = f(x) = c + (d - c)\left(1 - \backslash exp\left(-\backslash exp\left(b\backslash log(x)\backslash log(e)\right)\right)\right)$$

c

$$y = f(x) = c + (d - c)\backslash exp\left(-\backslash exp\left(b\backslash log(x)\backslash log(e)\right)\right)$$

Figure 2.2: a. The models representing a log logistic dose response (black) a steep slope approaching the lower asymptote: **Weibull-1 model** (Blue) and a steep slope leaving the upper asymptote associated with a **Weibull-2 model** (Red) adapted from (Ritz & Streibi, 2012). **b. Equation 2.2:** The Weibulls 2 equation. **c. Equation 2.3:** the Weibull's 1 equation. Both equations describe the relationship between plant response y and herbicide dose x. The four parameters are c- the lower limit, d- the upper limit (c and d values are fixed at 0 and 1 for a two parameter model, respectively), *b* the slope, and e the inflection point.

The resistance index was calculated using equation 2.4 which quantifies the level of resistance in a putative resistant population compared to a standard sensitive population. A population was considered to be resistant if the resistant index was greater than two.

$$RI = \frac{LD_{50}\ of\ the\ putative\ resistant\ population}{LD_{50} of\ the\ standard\ sensitive\ population} \quad (2.4)$$

2.2.4.2 Biomass data

A log-logistic 3 parameter dose response model (Equation 2.1) was fitted to biomass data. Regression diagnostics were performed to ensure the data conformed to the assumptions and if required; a BoxCox transformation of the data was conducted. This transformation was conducted provided the model fit was not compromised. The dose response model allowed the determination of the dose at which the growth of the population was reduced by 50% (GR_{50}).

The lack-of-fit test determines the model fit of continuous data. Explicitly, it can determine whether the residual sum of squares associated with an ANOVA is significantly less than that of the corresponding dose response model. As with the previous model fit test, a non-significant P-value (> 0.05) indicates that the dose response model fit is as good, as an ANOVA. In this case, the dose response model is favored and thus used to describe the relationship between variables as a simpler model, with less parameters (Bates & Watts, 1988).

2.2.4.3 Simplification of models

Minimization of model parameter number was applied for all analyses. Initially models were fitted independently so that each population had discrete parameter values. Subsequently, models are fitted where values for individual parameters, for example the slope parameters, are constrained to a single common value. If model fits are no worse, the simplified model was selected to describe the data (section 2.3.1 for example).

The DCR manual provides further information on how to conduct a dose response analysis within R (Ritz & Streibi, 2012).

2.3 Results

2.3.1 Jealott's Hill Dose response

The data from the Jealott's Hill dose response experiment was analyzed using a two parameter log-logistic (LL.2) model, with a single slope parameter (figure 2.3). The goodness-of-fit test for the LL.2 single slope model, produced a P-value of 0.5481 (X^2_{19}= 17.618). However, the goodness-of-fit test for the LL.2 multiple slope parameter model, produced a lower P-value of 0.4675 (X^2_{16}= 15.794). These two models were not significantly different ($F_{8,5}$ = 1.47, LR = 3.29, P=0.3495) and there was no significant difference between individually estimated slope parameters (t_{16}, P < 0.05 for all comparisons). In conclusion, fitting the slope parameters independently did not enhance the model. As a single slope parameters was fitted to all population survival responses which occur in parallel, thus the LD_{50} values represents the population's herbicide response. Specifically, dose response models that fit slope

parameters independently to each populations exhibit different relative resistance measures depending on the LD value used to represent the data (for example LD_{50} or LD_{90}). The resulting dose required to kill 50 % of individuals (LD_{50}) values for the Renville, Dumont and Holloway populations are in table 2.1. All three Minnesota populations have LD_{50} values significantly higher than the PP1 LD_{50} value (Renville: Holloway; Dumont, t_{19}= -8.8143; -8.9222; -10.5675, P <0.001 all comparisons). Furthermore, Renville and Dumont populations are significantly more resistant than the Holloway population (t_{19}= 3.021; 2.154, P< 0.01; <0.05, respectively) but there was no significant difference between the Renville and Dumont population. The resistance indices (RI) were 4.5, 5.6, and 6.6 for Holloway, Dumont and Renville, respectively. All resistant indices are greater than two indicating that glyphosate resistance was present within the three populations from Minnesota. Therefore the Dumont, Holloway and Renville populations are resistant to glyphosate when compared to the PP1 sensitive population and all three populations can be classified as resistant.

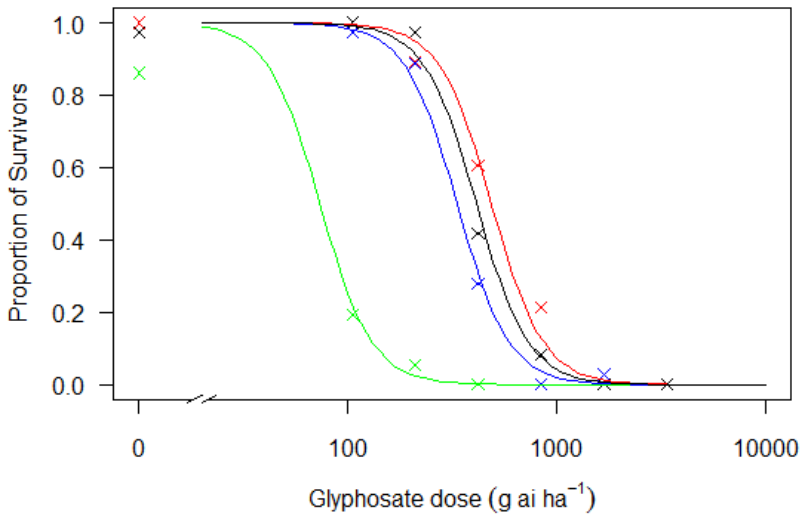


Figure 2.3: Proportional survival of four *A. tuberculatus* populations at increasing doses of glyphosate: Renville (-x-), Dumont (-x-), Holloway (-x-) and a standard sensitive PP1 (-x-). The data points are the observed values and the log-logistic 2 parameter models are denoted by the fitted lines (a single slope parameter fitted for all populations).

Table 2.1: Parameter estimates for the Jealott's Hill log- logistic two parameter dose response model. The slope of the relationship (b) was 3.50 (± 0.301) for all populations. Values in brackets are standard errors.

Population	LD_{50}	RI
PP1	73.52 (8.342)	-
Dumont	411.86 (30.417)	5.60
Holloway	333.28 (24.819)	4.53
Renville	486.63 (40.883)	6.61

2.3.2 Wellesbourne dose response

2.3.2.1 Wellesbourne dose response: survival

The second dose response experiment examined the resistance levels within the Renville and Dumont populations when compared to the S2 sensitive population. A two parameter log-logistic model was fitted to the data. The goodness-of-fit tests were performed and the results dictated that the slope parameters should be estimated independently for each population (figure 2.4). The LD_{50} and resistant index values for Renville, Dumont and S2 populations are in table 2.2. The goodness-of-fit test for the two parameter log-logistic model signified a satisfactory model fit (X^2_{12}= 16.896, P = 0.1536).

The resistance indices are 3.42 and 3.22 for the Renville and Dumont populations when compared to the S2 sensitive population of *A. tuberculatus,* respectively (table 2.2). These resistance indices are greater than two, therefore the Renville and Dumont populations of *A. tuberculatus* are resistant to glyphosate when compared to the S2 sensitive population. Renville and Dumont LD_{50} values are significantly different to the S2 sensitive population (P <0.001 both models, t_{12} = 11.515; 9.876 respectively). As in the Jealott's Hill dose response experiment there was no significant difference between the Renville and Dumont LD_{50} values.

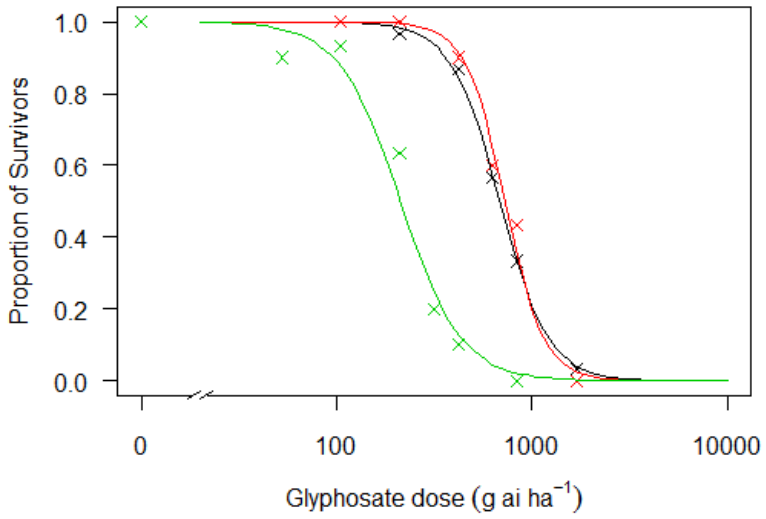


Figure 2.4: Proportional survival of three *A. tuberculatus* populations at increasing doses of glyphosate: Dumont (-x-), Renville (-x-) and S2 sensitive (-x-). The data points are the Wellesbourne dose response experiment means of the observed values and the log- logistic two parameter models are denoted by the fitted lines.

Table 2.2: Parameter estimates for the survival Wellesbourne dose response data for the log- logistic two parameter model. The parameters: b = slope, LD_{50} – lethal dose required to kill 50% of the population. RI = resistant index (based on the LD_{50}) compared to S2 sensitive. Values in brackets are standard errors.

Population	*b*	LD_{50}	RI
S2	2.78 (0.402)	212.38 (16.974)	1
Dumont	3.48 (0.573)	683.29 (44.554)	3.22
Renville	4.33 (0.765)	726.70 (41.312)	3.42

2.3.2.2 Wellesbourne dose response: biomass

The fresh biomass data for the second dose response reveals a similar trend to that observed in the survival data. A three parameter log-logistic model was fitted to the data (figure 2.5). Goodness-of-fit tests were performed to confirm that the slope parameter and upper limit should be estimated independently. The three parameter log-logistic model was the optimal model indicated by the lack-of-fit test, ($F_{609,621}$= 1.524; P =0.1107). The comparison of resistant and standard sensitive population GR_{50} values produced resistance indices of 6.31 and 8.67, for Dumont and Renville populations, respectively (Table 2.3). The Dumont and Renville GR_{50} values are significantly different to the S2 sensitive population (P <0.001 both models, t_{621} = 11.17; 6.62, respectively). As with the LD_{50}, there was no significant difference between the GR_{50} of the Dumont and Renville populations.

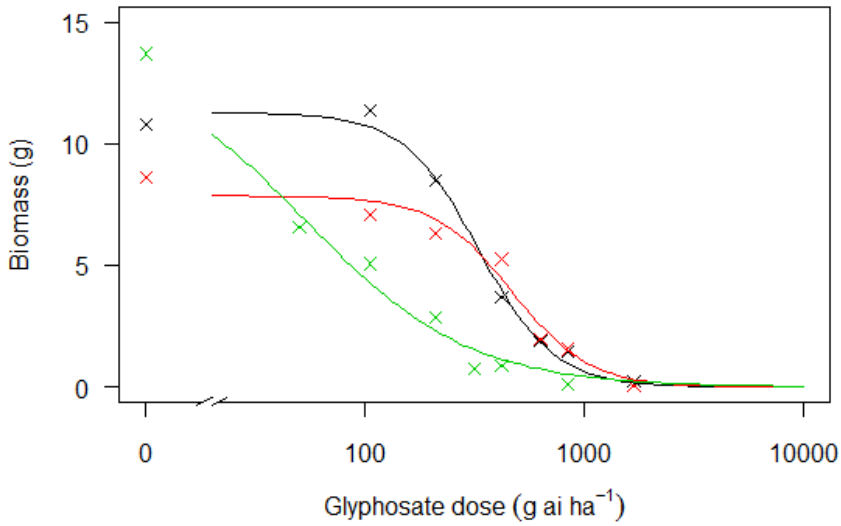


Figure 2.5: Biomass of three *A. tuberculatus* populations at increasing doses of glyphosate. The log- logistic three parameter model for the Wellesbourne dose response experiment. The data points are observed mean biomass and the log-logistic, 3 parameter models are denoted by the fitted lines, Dumont (-x-), Renville (-x-) and S2 sensitive (-x-).

Table 2.3: The log-logistic three parameter model Wellesbourne dose response parameter estimates. The parameters: *b* =slope, *d* = upper asymptote, GR_{50} = dose required to reduce growth rate by 50% and RI = resistant index (based on the GR_{50}) compared to S2 sensitive. Values in brackets are standard errors.

Population	b	d	GR_{50}	RI
S2	1.17 (0.139)	13.64 (0.543)	53.89 (7.33)	-
Dumont	2.54 (0.326)	11.27 (0.438)	330.43(23.63)	6.31
Renville	2.46 (0.813)	7.85 (0.529)	467.02(61.92)	8.67

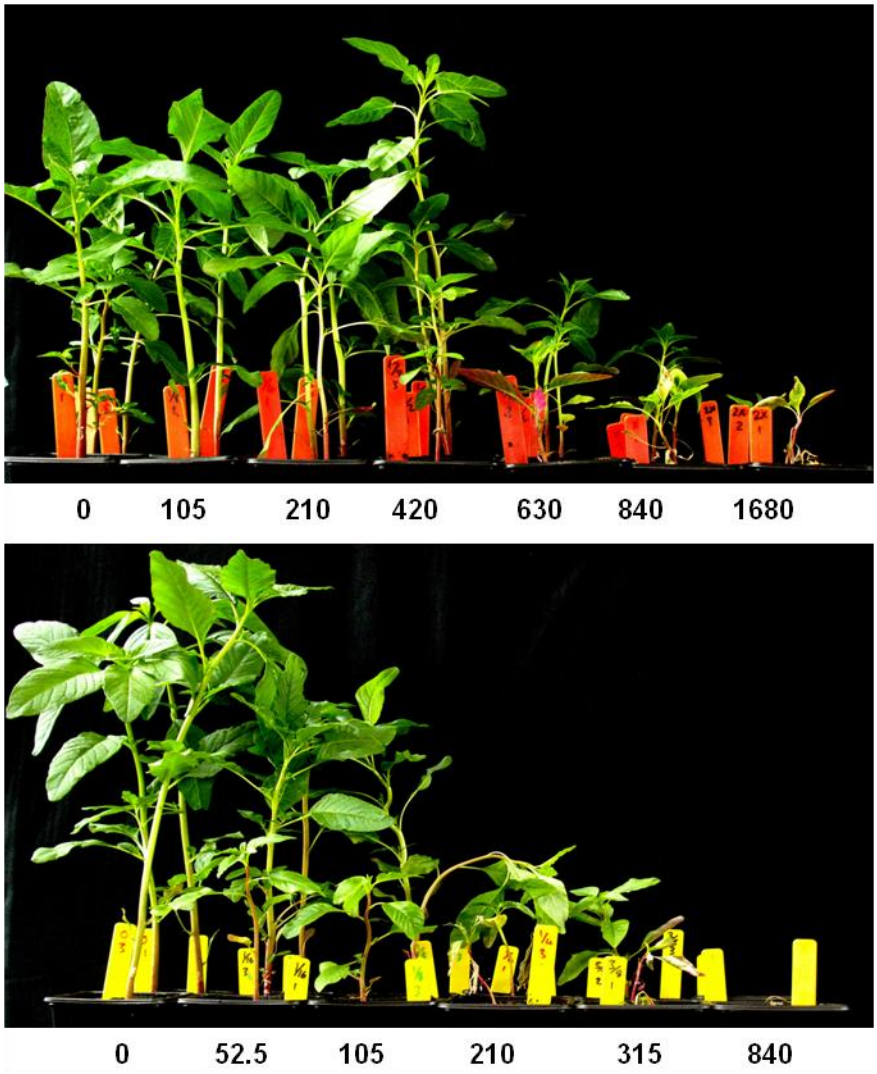


Figure 2.6: Photos of Wellesbourne dose response plants S2 sensitive population (above) and Dumont population (below). The numerical values indicate the glyphosate treatment applied to the plants in g ai ha^{-1}.

2.4 Discussion

2.4.1 Glyphosate resistance levels in the Minnesota populations

The Jealott's Hill dose response experiment produced resistance indices (based on the LD_{50} produced by mortality data) of 6.61, 5.60 and 4.53 for the Renville, Dumont and Holloway populations, respectively. The Wellesbourne dose response experiment produced resistance indices of 3.42 and 3.22 for the Renville and Dumont populations, respectively; when compared to a S2 sensitive population. All resistant population LD_{50} values were greater than two. In conclusion Renville, Dumont and Holloway populations were resistant to glyphosate.

Speculation by Shaner, (2010) hypothesized that the level of resistance observed in a weed population measured through a dose response experiment can indicate the type of resistance mechanism present within a population (Shaner, 2010). Shaner, (2010) suggests that a 3 to 5 fold resistance level is indicative of an *EPSPS* target-site mutation mechanism of resistance whereas a 5-15 level of resistance is indicative of reduced translocation (Shaner, 2010). Therefore, it may be hypothesized that the resistant indices exhibited in the Wellesbourne and Jealott's Hill dose response experiment indicate that glyphosate resistance may be caused by a target-site mutation rather than mechanisms associated with impaired translocation.

In the Jealott's Hill dose response experiment, the fitted model (figure 2.3) indicates that 30.3, 25.3 and 19.7 % of plants would survive at field rate application (840 g ai ha^{-1}) in the Renville, Dumont and Holloway populations, respectively. Furthermore, the fitted model for the Wellesbourne dose response experiment (figure 2.4),

indicates that the Renville and Dumont populations had 43.2 % and 42.2 % survival at field rate application, respectively. Therefore, in an agricultural field without the addition of an effective, non-glyphosate based weed control method; many of the individuals from the Dumont, Renville and Holloway populations would have survived under a glyphosate resistant crop production regime. This assumes a good level of glasshouse to field results translation. Indeed, the level of glyphosate resistance in a population of *A. palmeri* was measured in dose response experiments under field and glasshouse growth conditions. The resulting field LD_{50} values were approximately two times the values observed in the glasshouse (Culpepper *et al.*, 2006 cited in Shaner, 2010). When a dose response experiment is conducted within the laboratory it cannot fully represent the field response of a weed population as lower levels of weed control are anticipated within a field environment. Uniform pesticide application cannot occur across a field as many weeds will receive a lower herbicide dose due to sheltered weeds within refuges or under other vegetation (Gardner *et al.*, 1998). Furthermore, some weeds will be exposed to no herbicide application if germination occurs after pesticide application and finally application may occur at different life history stages to result in a variable herbicide response (Gardner *et al.*, 1998).

The LD_{50} of the Renville population has increased from 487 to 727 g ai ha^{-1} between the two dose response experiments and the LD_{50} for the Dumont population has increased from 412 to 683 g ai ha^{-1}. The Wellesbourne LD_{50} values were approximately 1.51 and 1.66 times greater than the corresponding Jealott's Hill LD_{50} values, for the Renville and Dumont populations, respectively. The Dumont and Renville LD_{50} values vary proportionally between the two experiments and thus the

consistency between the two data sets indicates that the Wellesbourne and Jealott's Hill dose response experiments provide a valid measure of resistance. The LD_{50} value for the PP1 standard sensitive population was 73.52 g ai ha^{-1} whereas the LD_{50} for the S2 sensitive population was 238.21 g ai ha^{-1}. The LD_{50} for the S2 sensitive population is 3.24 X the PP1 standards sensitive, this increase was greater than the ratio of the resistant populations between the two dose response experiments. The PP1 and S2 standard sensitive populations have different environmental histories, therefore one may hypothesise that the sensitive populations are genetically divergent and so will exhibit independent dose responses to glyphosate.

Generally, it is difficult to achieve repeatability between dose response assays. These difficulties can be attributed to the multiple variables which impact the efficacy of herbicide application. Indeed, a study conducted by Medd et al. (2001) looked at 59 dose response experiments conducted on *Avena spp.* using clodinafop-propargyl. It was found that spray volume, soil moisture deficit, cold stress and maximum temperature on the day of application all interact to affect the efficacy of the herbicide (Medd *et al.*, 2001).

2.4.2 Comparison with glyphosate resistant populations

Glyphosate resistant *A. tuberculatus* populations have evolved and established in multiple states: Illinois, Kansas, Iowa, North Dakota, South Dakota, Indiana, Missouri, Minnesota, Mississippi, Texas, Tennessee, Oklahoma and Nebraska (Heap, 2013). Generally, the level of glyphosate resistance found in weed populations is of a relatively low magnitude, with resistance indices varying from 3-15 (Shaner, 2010; Heap, 2013). These glyphosate resistance levels are modest when

compared to the resistance levels associated with alternate herbicidal modes. For example, weeds containing a target-site based resistance to acetolactate synthase herbicides often have resistance indices of between 100 and 1000 (Shaner, 2010; Tranel *et al.*, 2011; Heap, 2013). The levels of glyphosate resistance found in the *A. tuberculatus* Minnesota populations exhibited resistant indices of a similar magnitude to that reported by Tranel *et al.*, (2011). Thus, the reported levels of resistance in the *A. tuberculatus* Minnesota populations were of a relatively modest magnitude compared other herbicide resistance populations and thus support the observations of Shaner (2010).

A population of *A. tuberculatus* from Everly, Iowa had a resistance index (based on the LD_{50}) of 2.7. Therefore the Everly population contained a similar magnitude of tolerance to the Minnesota populations. However, the Everly RI was not reported as significantly different to the sensitive control and therefore the Everly population was not classified as resistant, unlike the Dumont and Renville populations (Zelaya & Owen, 2005). Interestingly, the sensitive control in the Everly dose response had an LD_{50} equal to the S2 sensitive control utilised in the Wellesbourne dose response experiment (0.21 kg ai ha^{-1}) (Zelaya & Owen, 2005).

A. tuberculatus plants from fields in Monticello; Missouri and Sutter; Illinois, were treated with 0.84 kg ai ha^{-1} of glyphosate to produce 10 and 15.3 % plant survival respectively (Smeda & Schuster, 2002). A dose response experiment must be conducted on Monticello and Sutter populations to allow a comprehensive comparison of resistance levels. However, it is clear that the Renville and Dumont

populations showed a predominately higher level of resistance to that seen in the Monticello and Sutter populations.

The GR_{50} values for Renville and Dumont *A. tuberculatus* populations are 0.47 (± 0.62) and 0.33 (± 0.24) kg ai ha^{-1} of glyphosate. By comparison two *A. tuberculatus* populations from Missouri, had GR_{50} values of 2.36 (± 0.24) and 1.16 (± 0.02) kg ai ha^{-1} of glyphosate (Legleiter & Bradley, 2008). These values are higher than the Renville and Dumont population indicating that the Missouri populations had a lower level of glyphosate induced growth reduction compared to the Minnesota populations.

2.4.3 Management of glyphosate resistance A. tuberculatus

The presence of glyphosate resistance in three populations of *A. tuberculatus* indicates that glyphosate application will not completely control the Renville, Dumont and Holloway weed populations. Therefore additional weed management tactics must be applied in glyphosate tolerant crop production systems, to control glyphosate resistant *A. tuberculatus.* Furthermore action must be taken to prevent the spread of resistance and reduce the population size of glyphosate resistant *A. tuberculatus* populations. The full extent of the negative implications of a widespread glyphosate resistant *A. tuberculatus* epidemic is still to be seen.

2.4.4 Conclusion

The Renville and Dumont populations exhibit significantly higher LD_{50} and GR_{50} values when compared to the PP1 and S2 standard sensitive population. The Holloway population exhibited a comparatively lower level of resistance (table 2.1)

and therefore was not utilised in the Wellesbourne dose response experiment. Therefore Renville and Dumont populations have been selected for phenotype segregation experiments.

In conclusion, a dose response experiment has confirmed the presence of glyphosate resistance within three populations of *A. tuberculatus* from Minnesota. The level of glyphosate resistance in the Renville, Dumont and Holloway populations was substantially greater than the PP1 and S2 sensitive populations. Subsequent to resistance quantification, Dumont and Renville populations have been selected for resistant and susceptible seed material production.

3.0 The method of segregating resistant and susceptible phenotypes from field populations to provide seed material for fitness cost experiments

3.1 Introduction

3.1.1 Fitness experimental material

It is imperative that the background genetic variation between resistant (R) and susceptible (S) fitness experimental material is minimised (Vila-Aiub *et al.*, 2011). The control of background genetic variation, allows fitness discrepancies between R and S phenotypes to be attributed to the polymorphisms at resistance alleles as opposed to non-resistance alleles (Bergelson & Purrington, 1996; Jasieniuk *et al.*, 1996; Vila-Aiub *et al.*, 2011). If the R and S experimental material has been sourced from two geographically isolated populations then inter-population variation will be present at multiple fitness-related alleles. Primarily, this genetic variation results from the local adaptation of populations to the differences in biotic and abiotic selection pressures between locations (Keller & Kollmann, 1999; Vila-Aiub *et al.*, 2011). Inter-population genetic variation may also result from inbreeding depression, linkage disequilibrium and non- random mating (Vila-Aiub *et al.*, 2011). Inbreeding depression can cause inter population variation in small and isolated populations due to the random loss or fixation of alleles by genetic drift (Keller & Waller, 2002). Moreover, variation in population structure can be caused by non-random mating between individuals due to the reproductive isolation, either by distance (Spatial) or flowering time (Temporal). Finally, differences in linkage disequilibrium between

two populations may result from discrepancies in the non-random association of alleles such that alleles co-occur at higher frequencies than predicted by Hardy-Weinberg principal.

In summation, isolating R and S phenotypes from a single population will minimise the background genetic variation between phenotypes (Neve, 2007). Therefore, when experiments compare phenotypes segregated from the same population, any observed fitness discrepancies can be attributed to resistance differences in the associated alleles (Neve, 2007).

3.1.1.1 The impact of Linked alleles

Linkage occurs when allele associations are maintained due to a low recombination frequency between two alleles on the same chromosome, resulting in the co-segregation of two alleles. For example, the introduction of the tobacco mosaic virus resistance gene, into multiple *Lycopersicon esclentum* crop cultivars was associated with substantial linked DNA (4 to 51 cM) after multiple rounds of backcrossing (Young & Tanksley, 1989). Indeed, the segregation of herbicide R and S phenotypes from a polymorphic population can result in the co-segregation of resistance-linked allele(s). Any resistance-linked alleles may incur a fitness cost, which will be equivalent to a resistance cost; whilst the resistance and linked-alleles remain associated (Purrington, 2000). However, in the presence of a fitness cost there will be a strong selection pressure for the resistance and linked alleles to disassociate (Purrington, 2000). If such a resistance allele association was found in *A. tuberculatus*, an out crossing species, there would be a high tendency for linkage

disassociation to occur, particularly when compared to an autogamous plant species, such as *A. thaliana* (Mauricio, 1998).

3.1.2 Methods used for phenotype segregation

To produce seed material for fitness analysis R & S phenotypes must be identified, segregated and bred to produce R and S seed for comparison. Identification of R and S phenotypes may be achieved through multiple methods (table 3.1). R parental plants may be identified through the application of the selective herbicide. However, if the genetic basis for resistance has not been determined, a fundamental difficulty is associated with susceptible phenotype identification. Namely, when a selective herbicide is applied to a susceptible plant, by definition, the herbicide will have a lethal impact. Therefore, even though the susceptible plant has been identified, deceased plants cannot be utilised for seed production. This fundamental contradiction has resulted in the creation of multiple methods to achieve non-destructive phenotypic identification.

Table 3.1: Segregation methods to produce glyphosate R and S seed material

Segregation Method	Description of method	Efficacy/ Applicability
Application of glyphosate	High rates of glyphosate may be applied to distinguish R plants and low rates to distinguish S plants. The absence of glyphosate susceptibility symptoms indicates R plants, whereas high susceptibility symptoms indicates S plants	Only a single discriminating dose can be used to determine the phenotype of plants therefore the dose must be carefully selected to avoid misclassification (Shaner, 2010). The most susceptible plants are killed as a result of glyphosate application, therefore dead plants cannot be selected for seed production.
Shikimate leaf disc assay	Detection of leaf shikimate accumulation (proxy for resistance) in the presence of glyphosate (Shaner *et al.*, 2005). (Wakelin & Preston, 2006; Culpepper *et al.*, 2006; Gaines *et al.*, 2010).	Minimal injury to parental plants. However, cannot detect resistance associated with impaired glyphosate translocation (Feng *et al.*, 2004; Shaner *et al.*, 2005; Nandula *et al.*, 2008).
Seedling segregation	Seedlings placed in low dose of glyphosate and assessed for susceptibility symptoms. Seedlings are recovered from the growth medium and grown up for breeding.	Rapid screening with low effort and cost. However, cannot detect resistance which is not apparent at a seedling stage (Shaner, 2010). Poor segregation of R and S *A. tuberculatus* plants observed after multiple rounds of selection (Zelaya & Owen, 2002)
Genotyping	Single nucleotide polymorphism analysis of the *EPSPS* (or alternate) alleles allows a genotype to be assigned to each individual (Menchari *et al.*, 2008; Wang *et al.*, 2010).	Ideal for target-site SNPs. However, the glyphosate resistance mechanism must be characterised at the gene level before genotyping can be utilised.
Vegetative propagation	Vegetative clones are propagated from parental plants and treated with discriminating glyphosate doses (Vila-Aiub *et al.*, 2005).	Multiple treatment doses may be applied to phenotype a single parental plant (Shaner, 2010). Clones can be propagated from offshoots of *A. tuberculatus* plants (Zelaya & Owen, 2005).

3.1.2.1 Segregation approach

As the resistance mechanism present in the Renville and Dumont populations was not initially known, the segregation method utilised for plant identification must work at the phenotypic level. Therefore, the shikimate assay and genotyping could not be used to distinguish R and S plants. In this experiment the vegetative propagation segregation method was used to identify R and S individuals for seed material production. Additionally, the application of glyphosate was utilised to identify R phenotypes to produce a second set of resistant seed families.

3.1.3 The impact of mechanism on segregation

The genetics of the resistance mechanism will have a large impact on segregation efficacy: a target-site mutation which is controlled by a single allele will segregate based on Mendelian inheritance (Mendel, 1986). However, a polygenic resistance mechanism will follow a complex inheritance pattern, and therefore multiple selection rounds may be required to produce distinct R and S seed lines (Zelaya & Owen, 2002a). Each selection for R and S phenotypes from a polygenic trait will select the extreme individuals at the tails of a resistance distribution and thus promote enhancement of the resistant or susceptible phenotype level in selected progeny. Two independent research groups have attempted to select a glyphosate resistant *A. tuberculatus* seed line from a glyphosate resistant agricultural population. In each case, multiple rounds of selection were conducted, however, although resistant individuals were identified, neither research groups could segregate a pure glyphosate resistant line (Tranel *et al.*, 2006). The difficulties associated with segregating resistant phenotypes may be attributed to a polygenic glyphosate

resistance mechanism in the *A. tuberculatus* study populations (Zelaya & Owen, 2005).

3.1.4 Objectives

The main objective of the chapter was to purify and phenotypically characterise resistant and susceptible seed material from the Dumont and Renville *A. tuberculatus* populations. This R and S seed material can thus be used for fitness cost experiments. To achieve the principal objective, multiple auxiliary objectives required completion:

- Determine discriminating doses for R and S identification.
- Phenotype identification through the application of the discriminating dose(s) to vegetative-clones and subsequent breeding of R and S plants with identical phenotypes.
- Quantification of resulting R & S seed material resistance levels through a dose response experiment to determine if complete R & S segregation has occurred.
- Validation of the vegetative-clone selection method for plant phenotype identification.

3.2 Methods

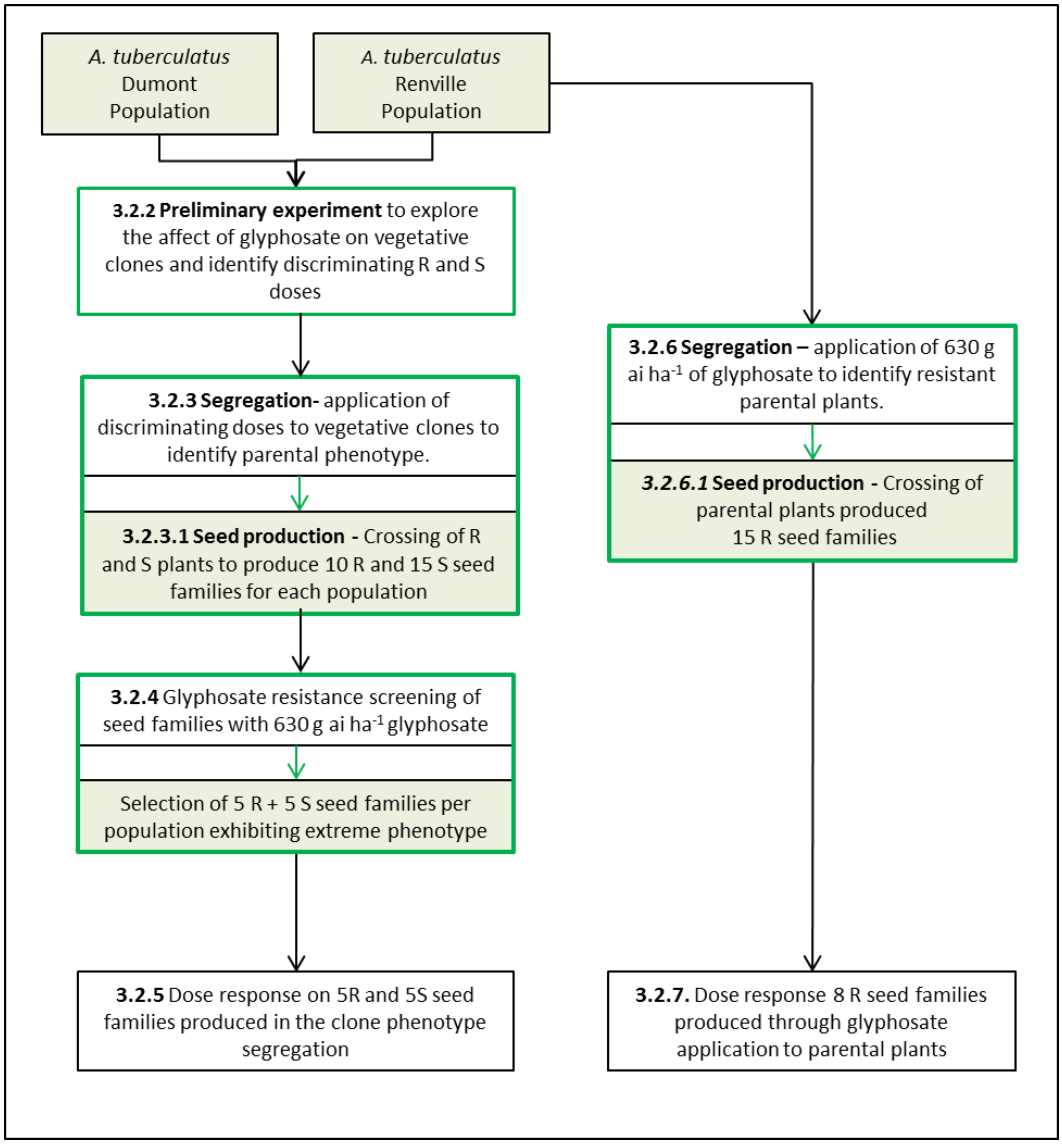


Figure 3.1: Scheme of segregation and dose response experiments. White boxes denote conducted experiments and green boxes denote the experimental material produced from preceding experiments and utilised in subsequent experiments. Numbers correspond to section numbers.

3.2.1 General protocols

3.2.1.1 Plant production

Seeds were sown onto leveled seed trays (15 x 20 cm) filled with fine grade sphagnum moss peat: sand; 24:1 (pH to 5.5 – 6.0; N = 150, P = 200, K = 200 mg L^{-1}; Levington growing media: FS2). Following sowing, seeds were covered in a 1 mm layer of compost and trays were watered. Seed trays were covered with aluminum foil and placed at 5 ^{o}C to break seed dormancy. After seven days, propagator lids were added and seed trays were placed in incubators at 24:18 ^{o}C (16:8 hr; day/ night), to optimise germination conditions. Seedlings were transplanted 14 days after germination. Seedlings were transplanted into pots filled with medium grade sphagnum moss peat 100 % (pH to 5.5 – 6.0; N = 200, P = 150, K = 200 mg L^{-1}; Levington growing media: M2) and placed in a glasshouse heated to 23/ 18 ^{o}C (16:8 hr; day/ night, with ventilation at +2 ^{o}C above set points), with supplementary lighting; unless otherwise specified. For screening and dose response experiments a specified seedling number was transplanted per pot and positioned to produce equidistant plant spacing. Atypically sized individuals were removed from pots before glyphosate treatment.

3.2.1.2 Herbicide Application

Herbicide application was conducted using a Berthoud Velmorel 200 pro knapsack sprayer and a Deflector Anvil Polijet nozzle (D/1.2/1). For each experiment the walking speed was 2 km hr^{-1}, set with a metronome and pressure was set at 200 kPa. The nozzle height was standardised at 40 cm above the plant canopy to deliver a

spray volume of 300 L ha^{-1}. Plants grown from seedlings were treated at the 6 to 8 leaf stage and vegetative clones were treated 18 days after transplanting. In each experiment, plants were treated with appropriate doses of the isopropylamine glyphosate salt.

3.2.1.3 Plant phenotype identification

Post herbicide application plant survival was assessed 21 days after treatment. Survival and mortality assessment was performed according to set criteria, to minimize the potential for experimenter bias. Conclusive symptoms of mortality were complete plant necrosis, apical meristem necrosis, and root system disintegration (such that the plant could be removed from the soil with minimal force). Above ground fresh biomass was measured on plants cut at soil level. A seven point glyphosate symptoms score was used to distinguish the level of plant glyphosate susceptibility (6= complete necrosis (100 %; dead), 5= major necrosis (99-67 %) damaged meristem and or root system (figure 3.2.c; dead), 4= major necrosis (99-67 %) unaffected meristem and root system (alive), 3= moderate necrosis (66- 33 %; alive), 2= minimal necrosis (33- 1 %)/ major chlorosis (>50 % ; alive), 1= minor chlorosis (<50 %; figure 3.2.b; alive), 0- No glyphosate symptoms (figure 3.2.a; alive).

Figure 3.2: Example glyphosate susceptibility score photos Numbers in brackets indicate the score of plants: a. no glyphosate symptoms (0) b. leaf chlorosis (1) c. major necrosis (5).

3.2.2 The effect of glyphosate dose on A. tuberculatus clones

A dose response experiment was conducted on vegetative clones to determine discriminating doses for R and S parental plant phenotype identification. The standard sensitive *A. tuberculatus* population (S2), Dumont and Renville plants were propagated as outlined in section 3.2.1.1. 30 plants per population were individually transplanted into 9 cm pots. The central apical growing stem was removed 8 weeks after germination to ensure sufficient branching for clone production. A high N: P nutrient solution was added to the soil medium of parental plants to discourage flowering. Cuttings were taken from below a leaf apex, the corresponding leaf was removed and the stem was implanted into moist FS2 soil medium (3.2.1.1). Pots containing cuttings were covered with polythene to maintain a humid environment

for 14 days, after which adventitious roots had formed (figure 3.3). Clones without root formation (which could be uprooted with minimal force) and dead plants were removed from the experiment. Seven clones were taken from each parental plant and each clone was sprayed with a different glyphosate dose. Renville and Dumont clones were treated with glyphosate at 0, 60, 120, 420, 540, 840 and 1680 g ai ha^{-1} whereas the S2 sensitive clones were treated with 0, 30, 60, 120, 180, 420 and 840 g ai ha^{-1}. Glyphosate was applied as outlined in section 3.2.1.2. The three populations (Dumont, Renville and S2 Sensitive) and seven glyphosate dose rates produced a total of 21 treatments. For each population, 30 replicate parental plants per treatment produced a total of 630 clones. Plants were maintained in a glasshouse heated at 25/ 20 ^{o}C (16:8 hr; day/ night, with ventilation at +2 ^{o}C above set points), with supplementary lighting. Measures of survival, glyphosate symptoms and above ground fresh biomass were taken 21 days after glyphosate treatment. The discriminating doses were selected based on glyphosate survival and glyphosate symptoms at each glyphosate treatment dose. Symptoms of chlorosis were exhibited in controls due to the propagation process, fortunately no necrosis was observed in the clones. Therefore, clone necrosis symptoms were used to determine the phenotype of parental plants in the subsequent R and S segregation.

Figure 3.3: Example photo of clones; three clones taken from a single plant, indicating root formation levels 12 days after transplanting.

3.2.3 Resistant and Susceptible segregation through cloning protocol

The segregation was conducted to achieve R and S phenotype identification of seed production plants from Renville and Dumont populations. Dumont and Renville seedlings were produced as outlined in section 3.2.1.1. A total of 265 seedlings per population were transplanted into 7 cm pots filled with medium grade sphagnum moss peat 100 % (pH to 5.5 – 6.0; N = 200, P = 150, K = 200 mg L^{-1}; Levington growing media: M2) and raised under glasshouse growing conditions of 25/ 20 ^{o}C (set heating temperatures; 16:8 hr; day/ night, with ventilation at +2 ^{o}C above set points), with supplementary lighting. At 3 weeks after transplanting plants were re-potted into 11 cm pots containing M2 soil medium (detailed above) and transferred to a polytunnel. Vegetative clones were propagated as outlined in section 3.2.2. Clones were removed from parental plants after substantial vegetative proliferation, 6 weeks after transplanting. The procedures for clone production, phenotype identification and selection are outlined in figure 3.4. Following clone propagation,

pruning of vegetative growth of parental plants was conducted to discourage plant flowering prior to clone phenotyping. When flowering did occur, terminal flowers were covered with bread bags to prevent pollination prior to phenotype identification. The optimum glyphosate segregation dose rates were determined based on the glyphosate symptoms observed in the vegetative clone dose response experiment (3.3.1). Clones were scored for survival, glyphosate symptoms and fresh weight at each of the glyphosate treatment doses as outlined in section 3.2.1.3. Susceptible parental plants were identified when complete necrosis of all clone tissue had occurred at the three glyphosate dose treatments. Conversely, resistant parental plants were identified when all clones survived the glyphosate dose treatments and exhibited no symptoms of necrosis or chlorosis.

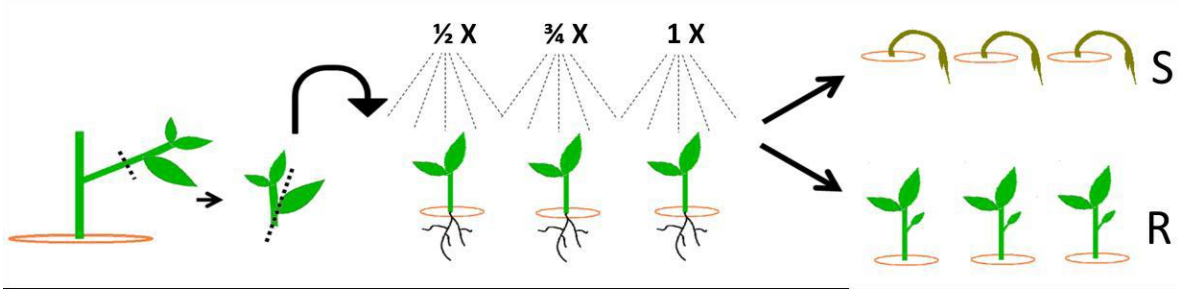


Figure 3.4: Scheme of clone propagation method for parental plant phenotyping. Three leaf cuttings were removed from individual parental plants and transplanted into pots to encourage rooting and re-growth. 18 days after propagation, three established clones per plant were treated with 420 (0.5x), 640 (0.75X) or 840 g ai ha^{-1} (1X) of glyphosate. Survival and growth was assessed and plants were scored as R or S depending on clone susceptibility symptoms at the three glyphosate doses.

3.2.3.1 Seed Production

The clone glyphosate screen (section 3.2.3) identified ten male and ten female resistant parental plants per population and 15 male and 15 female susceptible parental plants per population. Following phenotype identification all male and female plants with the same population-phenotype identity were placed inside a pollen proof cage in close proximity to ensure cross-pollination. The four crosses (resistant Renville, susceptible Renville, resistant Dumont and susceptible Dumont) were conducted inside a glasshouse without supplementary heating or lighting (average temperature: 16.72 ^{o}C (±4.72)). Male and female plants were arranged in a chequered square layout (figure 3.5) to maximise cross-pollination. After one month, female plants were transferred to a glasshouse compartment heated at 23/ 18 ^{o}C (16:8 hr; day/ night, with ventilation at +2 ^{o}C above set points), with supplementary lighting to optimize the seed maturation environment (Bell & Tranel, 2010). After two months, seed was harvested and dried at 15 ^{o}C, 15 % RH. All seed was harvested from each plant separately and maintained as discrete half sibling seed families. Seed was removed from branches manually and threshed. The material was shaken through 2.5 and 5 mm sieves to remove large plant debris and then placed in a seed aspirator to separate seeds from plant debris. The cleaned seed was stored at 5 ^{o}C, 5 % RH until use.

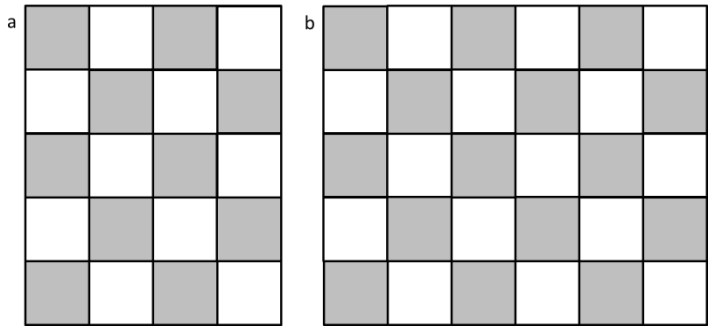


Figure 3.5: The layout of plants for a. resistant crosses and b. susceptible crosses. Grey squares represent male plants and white squares represent female plants. This layout minimised any discrepancies in the pollination distance between plants.

3.2.4 Glyphosate response of seed families from cloning protocol

A glyphosate response experiment was conducted to determine the extent to which the cloning, phenotype identification and bulk crossing had resulted in segregation of glyphosate R and S phenotypes across seed families produced in 3.2.3. Progeny from 15 Renville and 15 Dumont susceptible parental plants and 10 Renville and 10 Dumont resistant parental plants were selected with a single dose. Seeds from each maternal plant were maintained as half-sib seed families. Seedlings were produced as outlined in section 3.2.2.1, with 10 seedlings transplanted per 1.5 L pot. Four replicate pots of ten seedlings were produced for each of the 50 seed families, plus the S2 standard sensitive population to produce a total of 204 pots and 2040 plants. Glyphosate was applied at 630 g ai ha^{-1} (75 % of the field rate application) as outlined in section 3.2.2.2. Pots were completely randomized to account for any environmental differences within the glasshouse. Survival was recorded as specified in section 3.2.1.3.

3.2.5 Dose response of seed families from cloning protocol

The five most resistant and the five most sensitive seed families were selected for subsequent investigation based on the percentage survival of individuals at 630 g ai ha^{-1} observed in the glyphosate response experiment. A dose response experiment was conducted to quantify the resistance level in the 20 selected seed families. The Renville resistant seed families were: 1,315, 90, 226 and 285; Renville sensitive: 29, 141, 153, 21 and 289; Dumont resistant seed families: 255, 87, 321, 59 and 271 and Dumont sensitive: 187, 197, 97, 175, 80. In addition to the selected seed families, the S2 sensitive population was utilised as the standard sensitive control.

Seed family plants were produced as outlined in section 3.2.1.1. Ten seedlings were transplanted into three replicate 1.5 L pots for each seed family. Three replicate pots were produced for each of the seven glyphosate dose rates and 21 seed families to result in a total of 147 treatments and a total of 4410 plants. Plants were treated with 0, 105, 210, 420, 840, 1680, g ai ha^{-1} of glyphosate as outlined in section 3.2.1.2. A randomized block design was used. Plants were assessed through percentage survival and total plant fresh biomass per pot, as specified in section 3.2.1.3

3.2.6 Resistant segregation through glyphosate application to parental plants

An alternative means of segregating resistant plant material was attempted by directly applying 630 g ai ha^{-1} of glyphosate to seedlings of the Renville population. This segregation method was used to determine the validity of the vegetative-clone phenotype identification segregation method and to provide an alternative source of

segregated R seed material. Renville seed was germinated and re-potted as outlined in 3.2.2; 200 seedlings were transplanted into 9 cm square pots, one per pot. Surviving plants were re-potted and maintained in the glasshouse until flowering. The fifteen most resistant male and female plants were selected and bulk-crossed for seed production. Following phenotype identification, all male and female plants were arranged in a chequered square layout (figure 3.5b) inside a glasshouse compartment heated at 23/ 18 ^{o}C (16:8 hr; day/ night, with ventilation at +2 ^{o}C above set points), with supplementary lighting. Seed was harvested, cleaned and stored as outlined in 3.2.3.1.

3.2.7 Dose response of seed families from direct application protocol

A dose response experiment was conducted on eight seed families, which were randomly selected from those which had been segregated through glyphosate application to parental plant (3.2.7). The dose response was used to characterise the resistance level found within seed families 1, 2, 5, 7, 8, 9, 23 and 26 from the second resistant segregation. Plants were produced as outlined in section 3.2.1.1 with eight seedlings transplanted per 1.5 L pot. The dose response experiment was conducted as outlined in section 3.2.2.1 and 3.2.7; however, individual fresh biomass was measured for each plant, as specified in section 3.2.1.3.

3.2.8 Statistical analysis

Dose response analyses were conducted as outlined in section 2.2.4. For survival data two parameter, Log-logistic (Eq. 2.1), Weibull's type 1 (Eq. 2.3) and Weibull's type 2 (Eq. 2.2) dose response models were fitted independently to each seed family.

The model fit of each dose response model was determined using the goodness-of-fit tests. Subsequently, the best fitting model was selected to describe each seed family and determine parameters (3.3.3, 3.3.4)

The information collected from the dose response experiment on 20 seed families produced through the clone protocol can be utilised to determine whether a growth penalty is associated with resistance. The biomass of the control (upper asymptote of the dose response model), provided a measure of seed family growth in the absence of herbicide application whereas the GR_{50} provided a measure of seed family resistance. Thus, a Pearson's correlation analysis was conducted on the upper asymptote and GR_{50} parameters produced from the dose response analysis.

The Shapiro-Wilk test and Bartlett's test were conducted to determine whether data had a normal distribution and homogenous variance respectively. Comparative statistics were performed when required as listed below. If the data did not conform to the assumptions of a parametric test, a Mann- Whitney U test was conducted, however, if the data conformed to the assumptions an ANOVA was performed.

- The Mann-Whitney U test was conducted to determine whether the glyphosate treatment doses produced high glyphosate susceptibility symptoms in vegetative clones relative to the control (3.3.1).
- The Mann- Whitney U test was conducted to determine whether the proportional survival of individuals after glyphosate response was greater in seed families with resistant phenotyped parents (3.3.2)

- A t-test was conducted to determine whether the LD_{50} values for the putative R and S seed families produced through the clone protocol. Seed families were categorised as R or S based on parental phenotype (3.3.3).
- A t-test was conducted to determine whether the resistant indices for R seed families produced through the clone protocol differed from the resistant indices of the seed families produced through the glyphosate application protocol (3.3.4)

3.3 Results

3.3.1 The effect of glyphosate doses on A. tuberculatus clones

The clone glyphosate symptom score was utilised to determine the relative effect of glyphosate on vegetative clones. The 420 g ai ha^{-1} glyphosate treatment dose, exhibited a significant difference in clone glyphosate symptoms from the control, for Dumont ($P<0.05$, $U=307$, $n=30, 30$) and Renville ($P<0.001$, $U=217.5$, $n=30, 30$) populations. Additionally, the 420 g ai ha^{-1} glyphosate treatment dose was the lowest dose to produce complete necrosis symptoms in clones, with 2/30 and 3/30 clones exhibiting complete necrosis symptoms for Dumont and Renville populations, respectively. Consequently, 420 g ai ha^{-1} of glyphosate was used to identify highly susceptible parental plants in the vegetative clone segregation.

The 840 g ai ha^{-1} glyphosate treatment dose produced 14 % and 6.7 % of clones with no or very low glyphosate susceptibility symptoms (chlorosis) for Dumont and Renville populations, respectively. The 1680 g ai ha^{-1} dose was highly effective at controlling vegetative clones as all clones exhibited glyphosate susceptibility

symptoms. Therefore, the 840 g ai ha^{-1} glyphosate dose was selected to identify highly resistant parental plants. A third glyphosate dose of 630 g ai ha^{-1} was selected to confirm the resistant and susceptible statuses of parental plants in the vegetative clone segregation.

In conclusion, the selected glyphosate doses were 420, 630 or 840 g ai ha^{-1} of glyphosate. Three clones were propagated per parental plant to allow plants to be screened with the three doses. If the three clones showed complete necrosis at the three doses then the parental plant was classified as susceptible. However, if three clones show no symptoms of necrosis then the parental plant was classified as resistant.

3.3.2 Glyphosate response of seed families from cloning protocol

The segregation event identified 10 resistant and 15 susceptible seed families per population. The glyphosate response experiment was conducted to determine whether R & S segregation had been achieved. The percentage survival values at 630 g ai ha^{-1} of glyphosate, for seed families were between 41.7 and 0 % (figure 3.6). The resistant Renville seed families had a high average percentage survival compared to the sensitive seed families 18.3 % (± 4.05), 6.6 % (± 1.43), respectively. Similarly, Dumont resistant seed families had a high average percentage survival compared to the sensitive seed families 17.0 % (± 4.54), 9.6 % (± 3.31), respectively. Thus it is possible to calculate that the vegetative clone phenotype identification segregation method has produced seed families with the average difference between R & S survival of 11.7 % for Renville and 7.4 % for Dumont, a very low level of segregation.

When the seed families were combined based on parental phenotype and population, there was a significant difference between the survival of glyphosate R & S phenotypes for the Renville populations ($U= 26.5$, $P<0.05$, $n=15, 10$) however, there was no significant difference for the Dumont phenotypes ($U= 40.1$, $P= 0.22$, $n=15, 10$). This indicates that segregation was more successful between the R and S Renville seed families.

Contrary to parental plant phenotype; seed family progeny from the resistant parental lines Dumont 144 and Renville 207 exhibited 0 % survival at 630 g ai ha^{-1} of glyphosate. However, seed family progeny from Dumont susceptible parental lines 142 and 95 exhibited 36 % and 30 % survival, respectively. Therefore, the application of a single glyphosate dose found partial segregation of the resistance trait to reveal a complex pattern of resistance trait segregation.

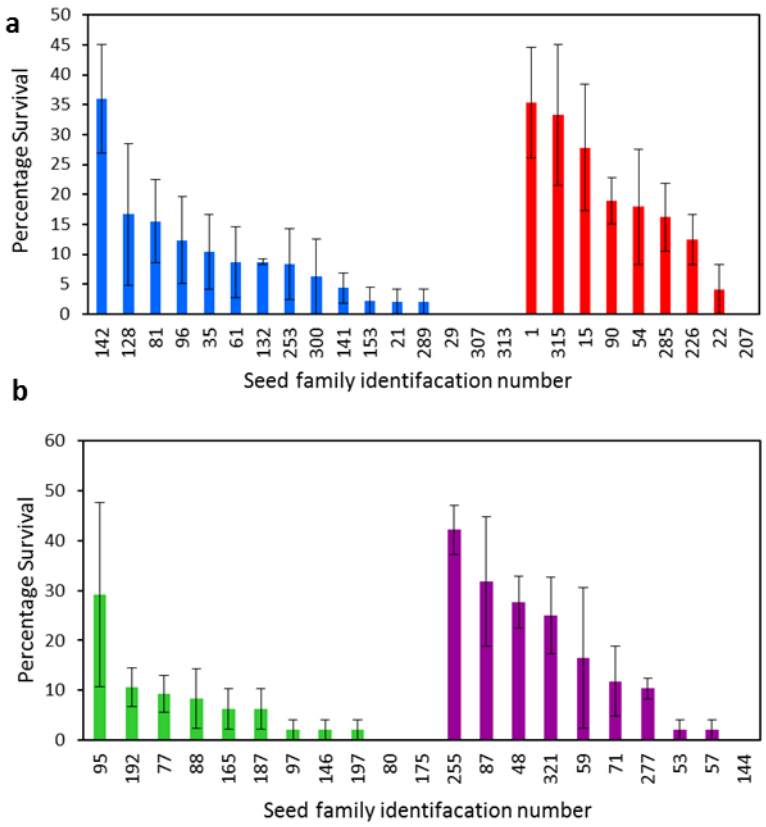


Figure 3.6: Percentage of plants per seed family that survived glyphosate application at 630 g ai ha^{-1}. a. Renville seed families, colours indicate the phenotype of parental plant: Blue- sensitive, Red- resistant. **b.** Dumont seed families, Green- sensitive Purple - resistant. Error bars are standard errors of the mean.

3.3.3 Dose response of seed families from clone protocol

The dose response analysis described the resistance level in 20 experimental seed families plus the S2 sensitive control population. The R and S LD_{50} values were subsequently used to determine whether substantial resistance segregation has been achieved in these families. The best fitting dose response model for each seed family (as measured by the goodness-of-fit test) are listed in table 3.2.

The LD_{50} values for the Renville seed families range from 109.0 (±25.29) to 882.5 (±96.78) g ai ha^{-1}. These values illustrate a broad resistance spectrum across the seed families. The resistant Renville seed families had significantly greater LD_{50} values than the susceptible phenotypes (t_8= 3.31, P< 0.05), and therefore seed families have segregated in accordance with the parental phenotypes (figure 3.7).

The LD_{50} values for the Dumont seed families range from 275.34(±22.110) to 600.43(±40.655) g ai ha^{-1}. The comparison of the LD_{50} values from the Dumont population R & S parental seed lines, showed that segregation did not occur in accordance with the parental plant phenotype (t_8= 0.63, P= 0.55)(figure 3.8). This analysis indicates ineffective segregation of the resistance trait in the R and S Dumont seed lines and thus Dumont seed families were not utilised in subsequent fitness experiments.

Table 3.2: The best fitting model for each of the Renville (R) and Dumont (D) seed families and the S2 sensitive population. The LD_{50} values and the resistant indices (RI) produced by each model are displayed and numbers in brackets are standard errors of the mean. All models are fitted with two parameters. The model type LL refers to log-logistic model (Eq.2.1), W1 Weibull's model has a steep slope approaching the lower asymptote model (Eq. 2.3) and W2 Weibull's has a steep slope leaving the upper asymptote (Eq. 2.2).

Seed fam.	Model type	Model fit P value	LD_{50}	RI
29R	W1	0.0539	335.55(40.40)	1.62
153R	LL	0.0864	356.63(41.91)	1.72
141R	W1	0.4031	457.67(37.61)	2.21
1R	W1	0.4845	882.47(96.78)	4.26
285R	LL	0.4615	628.92(69.53)	3.04
21R	W2	0.9392	343.92(34.15)	1.66
315R	W1	0.2794	620.49(65.89)	2.99
226R	W2	0.1412	401.73(33.83)	1.94
90R	W1	0.1546	648.03(79.80)	3.13
289R	W2	0.8418	109.02(25.29)	0.53
175D	W1	0.8697	532.66(37.254)	2.57
97D	W2	0.4263	600.43(40.655)	2.30
255D	W1	0.1166	776.01(75.257)	3.75
321D	W1	0.0897	553.85(39.921)	2.67
197D	LL	0.002	400.92(42.911)	1.93
80D	W2	0.7584	275.34(22.110)	1.33
87D	W2	0.0013	296.52(47.838)	1.43
59D	W1	0.0945	383.05(32.771)	1.84
277D	W2	0.4713	483.84(37.620)	2..34
187D	W2	0.0448	369.55(31.96)	1.78
S2	LL	0.9999	207.21(20.21)	-

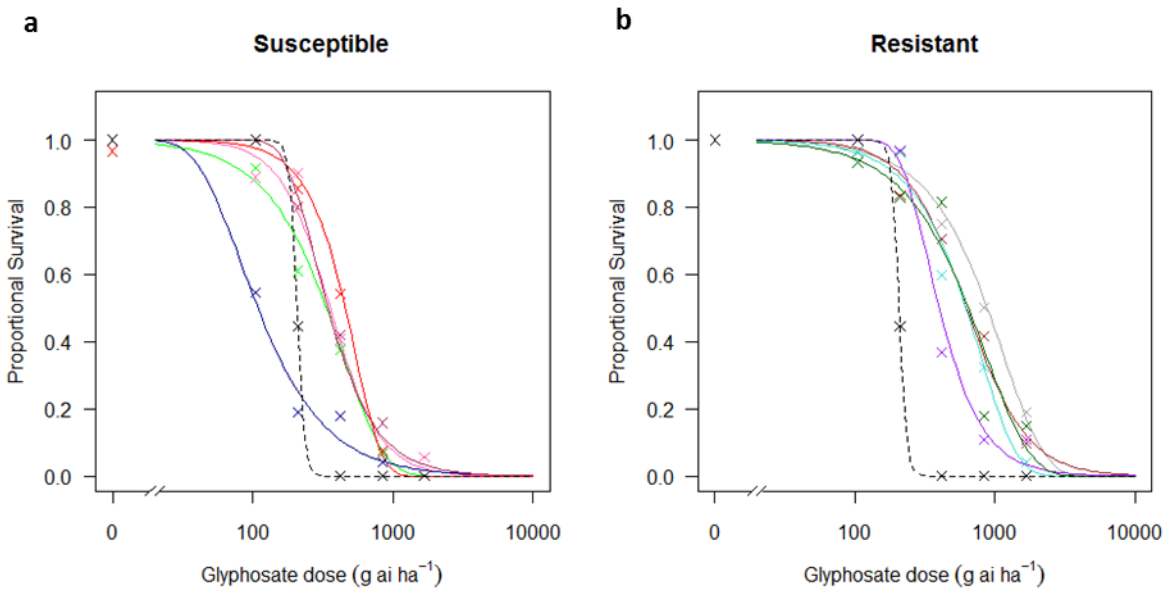


Figure 3.7: Renville susceptible (a) and resistant (b) seed family dose response models from the cloning segregation protocol. Black dashed lines denotes the S2 standard sensitive population, the colours correspond to seed family: Susceptible; 29 (-x-), 153 (-x-), 141 (-x-), 21 (-x-), 289 (-x-), Resistant; 1 (-x-), 285 (-x-), 315 (-x-), 226 (-x-), 90 (-x-), and S2 dashed (-x-). The data points are the seed family dose response experiment means of the observed values and the models are denoted by the fitted lines. Each seed family model has been fitted independently; see table 3.2 to view the model which has been fitted in each case.

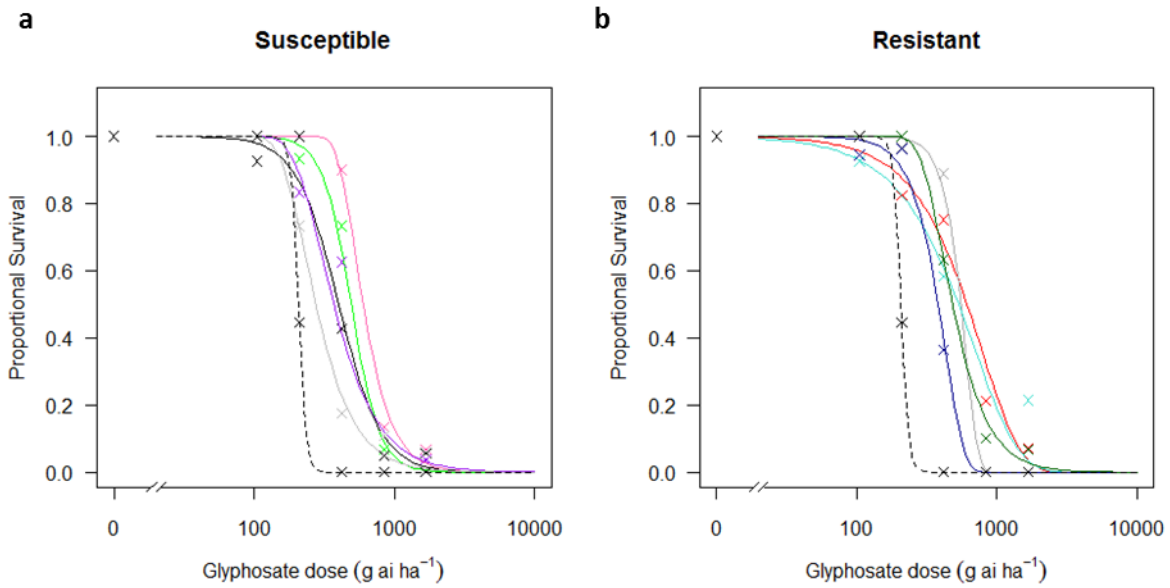


Figure 3.8: Dumont susceptible (a) and resistant (b) seed family dose response models from the cloning segregation protocol. Black Dashed lines are the S2 standard sensitive resistant seed family. The colours correspond to seed family: Susceptible; 187 (-x-), 197 (-x-), 175 (-x-), 97 (-x-), grey 80 (-x-), Resistant; 255 (-x-), 321 (-x-), 87, (-x-), 59 (-x-), 277 (-x-) and S2 dashed (-x-). The data points are the seed family dose response experiment means of the observed values and the models are denoted by the fitted lines. Each seed family models has been fitted independently, see table 3.2 to view the model type which has been fitted.

3.3.3.1 Fitness in biomass dose response

Fitness trade-off information can be extracted from biomass dose response models. The seed family dose response experiment investigated the resistance levels in ten Dumont and ten Renville seed families. The type 1 Weibull four parameter dose response model (with a lower asymptote estimated as a single value for all seed families) was applied to biomass data and used to estimate parameters (not shown). Correlation analysis indicated there was a negative correlation between GR_{50} and

intercept parameters across the 20 seed families (Pearson's correlation coefficient of 0.9247; P<0.05; df= 18; Figure 3.9). The negative correlation reveals that seed families with high herbicide induced growth reduction had a low biomass 49 days after transplanting in the absence of glyphosate. However, this relationship was not observed when the upper asymptote from the biomass dose response model was compared to the corresponding seed family LD_{50} values calculated in table 3.3.2. These results indicate that an increase in resistance may cause a growth penalty at an early vegetative phase in the absence of the stressor.

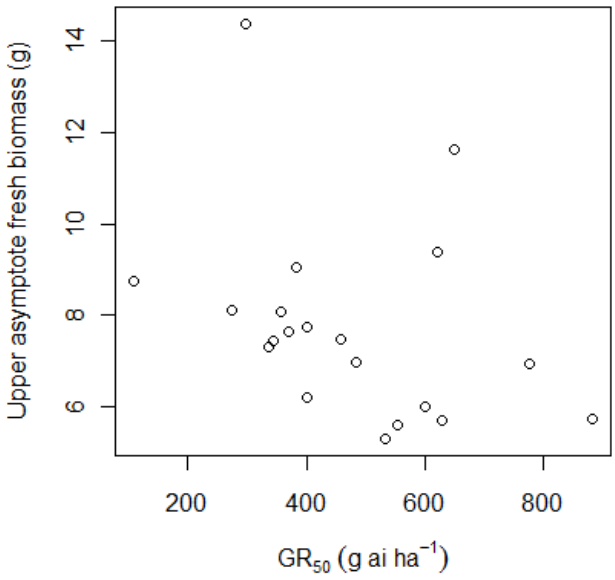


Figure 3.9: The association between GR_{50} and the upper asymptote of 20 from the Dumont and Renville seed families. Values are taken from the Weibull type one, four parameter model with a single lower asymptote parameter (analysis not shown). The r and P-values relate to a Pearson correlation analysis (df= 18).

3.3.4 Dose response of seed families from glyphosate application protocol.

The dose response analysis described the resistance level in eight of the experimental seed families produced in the second segregation (3.2.6) plus the S2 sensitive control population. The seed family resistant indices based on the LD_{50} values range from 1.4 (±0.22) for the most sensitive seed family to 4.3 (±0.69) for the most resistant seed family when compared to the S2 sensitive population (figure 3.10; table 3.3). The average resistance index for the second resistant selection was 2.8 (± 0.31), by comparison the average resistant index of R seed families produced through clone protocol was 2.7 (± 0.28). There was no significant difference between the resistant indices produced from plants selected in the two segregation events (t_{16}=0.19, P= 0.85). The lack of difference observed between the resistant indices from the two segregation protocols indicates that the two protocols segregate the resistance trait with equal efficacy. Therefore, the incomplete segregation observed in the clone segregation can be attributed to a quantitative resistance mechanism.

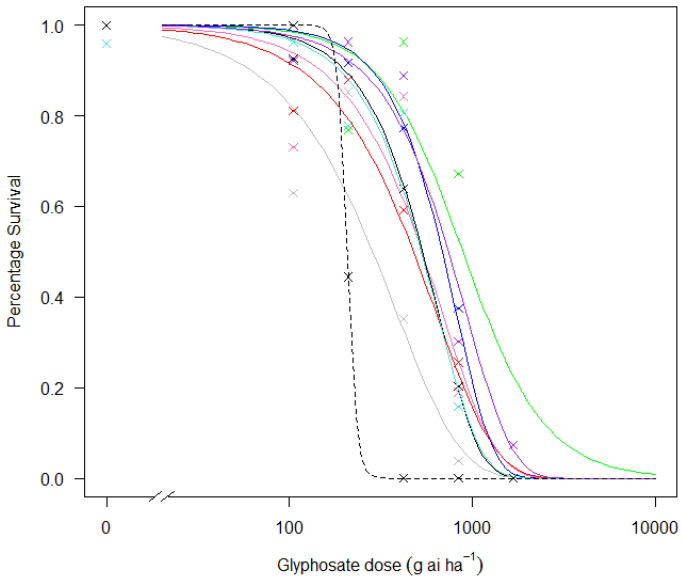


Figure 3.10: Renville resistant seed family dose response models for the second resistant segregation: S2 sensitive dashed (-x-), the colours correspond to seed family: 1 (-x-), 2 (-x-), 5 (-x-), 7 (-x-), 8 (-x-), 9 (-x-), 23 (-x-), 26 (-x-). The data points are the seed family dose response experiment means of the observed values and the models are denoted by the fitted lines. Each seed family model has been fitted independently; see table 3.3 to view the model which has been fitted in each case.

Table 3.3: The best fitting models for each of the second resistant selection seed-families using the dose response survival data, P-values indicates the fit of the models. The LD_{50} values and the resistant indices (RI) produced by each model are displayed and numbers in brackets are standard errors of the mean. All models are fitted with two parameters. The model type LL refers to log-logistic model (Eq.2.1), W1 Weibull's model has a steep slope approaching the lower asymptote model (Eq. 2.3) and W2 Weibull's has a steep slope leaving the upper asymptote (Eq. 2.2).

Seed family	Model	P-value	LD_{50}	RI
1	W1	0.1078	741.39(74.98)	3.58
2	W1	0.2574	685.65(65.82)	3.31
5	W1	0.1516	477.04(57.40)	2.30
7	W1	0.0004	531.35(58.83)	2.56
8	W1	0.0175	535.93(51.33)	2.59
9	LL	0	886.46(114.95)	4.28
23	W1	0.1830	285.22(37.24)	1.38
26	W1	0.5466	526.65(51.12)	2.54
S2	LL	0.9999	207.21(20.21)	-

3.4 Discussion

The clone dose response experiment allowed the selection of glyphosate doses of 420, 630 and 840 g ai ha^{-1} to phenotype plants through vegetative clone production. The vegetative clone method identified R & S plants for segregation crosses. These crosses produced seed families with incomplete R & S segregation and this was

attributed to a suspected quantitative resistance mechanism. The efficacy of the clone phenotyping method was verified through a second segregation whereby resistant parental plants were treated with glyphosate before survivors were crossed to produce resistant seed families. This glyphosate application method showed similar levels of segregation to the clone phenotyping method.

3.4.1 Incomplete segregation of seed families

The glyphosate response experiment found partial segregation of the resistance trait in the seed families produced through the clone protocol. Indeed, the average difference between R and S survival was 9.6 %. Therefore, the resistance trait was not controlled by a single locus, because when two phenotypically resistant plants are bred they will produce a minimum of ~75 % resistant individuals (under a conservative assumptions: two heterozygous (RS) parental plants) (Mendel, 1986). Dumont and Renville resistant phenotype lines have 17 and 18 % average progeny survival at 630 g ai ha^{-1}, respectively. The resulting low resistance levels may be attributed to an ineffective resistant plant identification method or a quantitative resistance mechanism. This complex pattern of resistance trait segregation was also observed in the glyphosate application segregation method. Indeed, similar seed family resistance indices were observed for the two segregation protocols indicating that the incomplete segregation may be attributed to a quantitative resistance trait.

3.4.2 Literature examples of incomplete segregation

Attempts to segregate glyphosate R & S phenotypes through multiple selection rounds, from an Iowa population of glyphosate tolerant *A. tuberculatus*, were not

successful (Zelaya & Owen, 2002a). The low segregation was hypothesised to be caused by a polygenic resistance mechanism, or the differential splicing of the *EPSPS* gene, generating distinct EPSPS isoforms (Zelaya & Owen, 2002a). Additionally, attempts were made to segregate a glyphosate resistant population of *A. tuberculatus* from Illinois, the segregation ratio did not coincide with that predicted for a single gene trait (Bell *et al.*, 2009). A similar pattern of low segregation was found in the Renville and Dumont segregation. The Renville seed family dose response did not find completely segregated R and S phenotypes. It is likely that multiple rounds of selection will be required to produce complete R and S seed lines from a polygenic resistant population (Zelaya & Owen, 2002a).

Incomplete R & S segregation has occurred in alternate glyphosate resistant weed species. Indeed, several segregation rounds were required before the complete segregation of R & S phenotypes in populations of *L. rigidum*, *E. indica* and *C. Canadensis* (Pratley *et al.*, 1999; Lee & Ngim, 2000; VanGessel, 2001). The incomplete segregation of the R and S phenotypes from a polymorphic population may be attributed to a pleiotropic fitness cost which is associated with a resistance allele or the presence of a polygenic resistance mechanism (Zelaya & Owen, 2002a).

3.4.3 Conclusion

No complete segregation was observed between the R & S seed families for the Renville and Dumont populations. This low segregation level led to the hypothesis that the causative resistance mechanism was quantitative. A secondary segregation confirmed that the Dumont seed families did not segregate based on parental phenotype; therefore the utilization of Dumont seed material was discontinued.

Conversely, the Renville seed families were confirmed to segregate based upon parental phenotype. The Renville seed family dose response experiment elucidated seed families with LD_{50} values ranging from 109.02 to 882.47 g ai ha^{-1}. Further to this, utilisation of seed families (half siblings) containing discrete LD_{50} values allows resistance costs to be studied across a phenotypic resistance spectrum. The Renville seed family numbers 1, 315, 90, 153, 141, and 29, (with the addition of 226 and 21 for glasshouse fitness cost experiment) were selected to form the experimental material for the fitness cost analysis in chapter 5 & 6.

4.0 The mechanism of glyphosate resistance in *Amaranthus tuberculatus* populations from Renville and Dumont

4.1 Introduction

It is important to study the fitness costs associated with resistance in the context of the causative resistance mechanisms. A mechanism-informed analysis will allow a fitness cost to be ascribed to the causative herbicide-resistance allele(s) within a species-specific context (Vila-Aiub *et al.*, 2009a). Four confirmed and two reputed mechanisms have evolved to endow glyphosate resistance or tolerance in 24 different weed species (Heap, 2012). Such mechanisms have evolved in an agricultural setting as a consequence of weed control strategies. The resistance mechanisms that have been characterised thus far are outlined below. In addition to this summary, for an extensive account of all glyphosate resistant incidences and mechanisms see the review by Beckie (2011).

4.1.1 Target-site resistance

4.1.1.1 Target-site alteration

One of the four elucidated glyphosate resistance mechanisms is due to a sequence mutation in the *EPSPS* target-site (Table 4.1). The predominant target-site modification that has evolved within an agricultural weed environment is a point mutation at the Pro-106 position to Ser, Thr, Ala and Leu. The Pro-106 mutation

occurs in the highly conserved region of the EPSPS sequence and narrows the active site to result in an increased dissociation constant for glyphosate. A high glyphosate dissociation constant conveys a reduction in glyphosate binding affinity (Stalker *et al.*, 1985; Padgette *et al.*, 1991; Funke *et al.*, 2009). The Pro-106 mutation confers glyphosate resistance in multiple weed species, whilst preserving EPSPS function (Beckie, 2011). Additionally, glyphosate resistance endowing target-site mutations have been selected for in *Escherichia coli* lab experiments, including, substitutions at the Gly-101 and Thr-102 locus', both of which convey a high level of resistance (Eschenburg *et al.*, 2002; Funke *et al.*, 2009; Powles & Yu, 2010). Fortunately, the Gly-101 and Thr-102 mutations severely reduce enzyme function. This high fitness cost may, perhaps, explain the absence of an established Gly-101 or Thr-102 EPSPS unaccompanied-mutation in existing agricultural weed populations (Powles & Yu, 2010). However, the double EPSPS Thr-102-Ile and Pro-106-Ser mutation has been found in *Eleusine indica* (goose grass). This double mutation conveyed a high level of resistance where 78% of individuals survived glyphosate treatment at 8640 g ai ha^{-1} (Jalaludin *et al.*, 2013). The Thr-102-Ile mutation was not found in the *E. indica* population in the absence of the Pro-106-Ser co-occurring mutation. This is concordant with the observation that an unaccompanied Thr-102 EPSPS mutation leads to reduced enzyme function (Powles & Yu, 2010; Jalaludin *et al.*, 2013). A Pro-182-Thr and Tyr-310-Cys double EPSPS mutation, has been found in a glyphosate resistant population of *Digitaria insularis* from Brazil (de Carvalho *et al.*, 2012). However, the latter experiment, selected the resistant and susceptible phenotypes from two discrete populations and thus, the observed polymorphism may not be associated with glyphosate resistance. Enzyme assays should be conducted to

confirm that the Pro-182-Thr and Tyr-310-Cys double mutant's displays reduced glyphosate binding affinity.

Table 4.1: Known glyphosate resistance endowing target-site *EPSPS* gene sequence mutations that have evolved in weeds following glyphosate selection in agricultural fields. Superscript letters link the incidence of resistance through the sequence mutation, the country of origin and the reference. *indicates a sequence alteration which requires further investigation.

Weed Species	Sequence alteration	Country	Reference
Eleusine indica (Indian goosegrass)	Pro-106 Ser[abc],& Thr[b] Pro-106-Ser + Thr-102-Ile[c]	Malaysia [ab]	(Baerson *et al.*, 2002)[a]; (Kaundun *et al.*, 2008)[ab] (Ng *et al.*, 2003)[ab] (Jalaludin *et al.*, 2013)[c] (Yu *et al.*, 2007)[c]
Lolium rigidum (Rigid ryegrass)	Pro-106 Ala[c], Thr[d], Ser[eg] & Leu[fg]	South Africa [cf] Australia [d] USA[e] Italy[g]	(Wakelin & Preston, 2006)[d] (Simarmata & Penner, 2008) [e] (Kaundun *et al.*, 2011) [f] (Collavo & Sattin, 2012)[g]
Lolium multiflorum (Italian ryegrass)	Pro-106 Ser[h], Ala[h] & Thr[i]	USA [i] Chile [h]	(Jasieniuk *et al.*, 2008) [i] (Perez-Jones *et al.*, 2007) [h]
Echinochloa colona (L.)	Pro-106 Thr[jl], & Ser[jkl]	Australia USA[jl]	(Thai *et al.*, 2012)[k] & (Alarcon-Reverte *et al.*, 2011)[j]
Digitaria insularis	Pro-182-Thr & Tyr-310 Cys*	Brazil	(de Carvalho *et al.*, 2012)
Amaranthus palmeri	Pro-106- Ser	USA	(Nandula *et al.*, 2013)

4.1.1.2 Amplification of target gene

EPSPS gene amplification is an alternative form of target-site alteration that endows glyphosate resistance (Gaines *et al.*, 2010; Mohseni-Moghadam *et al.*, 2013). *EPSPS* gene amplification leads to an increased number of *EPSPS* mRNA transcripts and EPSPS quantity. The increased production of EPSPS enzyme is able to out titrate the inhibition of glyphosate.

The first reported incidence of *EPSPS* gene amplification that evolved within an agriculture system was found in an *A. palmeri* population from Georgia, USA (Gaines *et al.*, 2010). The Georgia population evolved a 5 to 160 fold increase in *EPSPS* gene copies. When compared to susceptible individuals, this gene amplification produces up to a 35 fold increase in *EPSPS* gene expression levels. Furthermore, gene amplification levels were highly correlated with EPSPS protein expression levels in the absence of glyphosate application. This information indicates that all, or the majority, of the *EPSPS* gene copies associated with the resistant *A. palmeri* individuals were constituently expressed (Gaines *et al.*, 2010). Several populations of *A. palmeri* containing the glyphosate resistance gene amplification mechanism have since been reported in New Mexico (Mohseni-Moghadam *et al.*, 2013).

4.1.1.3 Target Gene Overexpression

EPSPS overexpression could potentially lead to increasing EPSPS protein concentrations and glyphosate resistance. Overexpression may be caused by a change in the promoter sequence of the target gene (Stefano *et al.*, 1980) or

epigenetic alterations such as DNA methylation, non-coding RNA and histone modification (Choudhuri *et al.,* 2010). However, the epigenetic modifications need to be heritable for the populations to adhere to the definition of resistance and for resistance to prove problematic within the field. The constitutive overexpression of *EPSPS* was detected (reputedly), in four glyphosate resistant populations of *Conyza canadensis* from the USA (Dinelli *et al.,* 2006) and two *Conyza bonariensis* populations from Spain (Dinelli *et al.,* 2008). The mechanism associated with overexpression in *C. canadensis,* produced a higher resistance factor than the populations' containing a resistance mechanism associated with impaired glyphosate translocation alone (Dinelli *et al.,* 2008). However, the two studies conducted by Dinelli *et al.,* (2006; 2008) do not investigate the potential for *EPSPS* gene amplification. Therefore gene amplification may have caused the increased *EPSPS* mRNA transcript levels and thus no conclusion can be made as to the causative mechanism of resistance. The level of gene expression may evolve to be induced by an external stimulus or trigger, such as herbicide application. However, inducible-expression has not been reported for *EPSPS* amplification or expression, to date.

4.1.2 Non-target-site mechanisms

4.1.2.1 Reduced uptake

Impaired glyphosate uptake can contribute to a weed population's glyphosate resistance level alongside a primary resistance mechanism (Michitte *et al.,* 2007; Nandula *et al.,* 2008). Impaired glyphosate uptake was found in resistant *L. multiflorum* populations from Mississippi, alongside the primary mechanism resulting in impaired translocation (Nandula *et al.,* 2008). This reduction in uptake

was not due to a thickening of the epicuticular wax layer but may be caused by a change in the composition of the waxy layer (Nandula *et al.*, 2008). Similarly, reduced glyphosate uptake was found in a population of *L. multiflorum* from Chile, alongside two additional resistance mechanisms. Here, reduced herbicide uptake was associated with a thicker abaxial surface layer and surface wrinkles in the resistant but not the susceptible phenotype (Michitte *et al.*, 2007).

4.1.2.2 Impaired translocation/sequestration

The phytotoxic effect of glyphosate relies upon glyphosate translocation through the phloem to the meristematic tissue. After translocation, glyphosate inhibits EPSPS which leads to the accumulation of shikimate and benzoic acid and a deficit in aromatic amino acids (Dekker & Duke, 1995). Notably, glyphosate resistant *A. tuberculatus* (Tall Waterhemp) individuals from Mississippi showed significantly lower glyphosate translocation; 23 % compared to 32 %; 48 hours after treatment for resistant and susceptible individuals, respectively (Nandula *et al.*, 2013). Furthermore, numerous populations of *C. canadensis* (Horseweed), *L. rigidum* (Rigid ryegrass) and *Sorghum halepense* (Johnsongrass) (Powles & Preston, 2006; Vila-Auib et al., 2007; Powels & Yu, 2010) have independently evolved mechanisms which result in restricted glyphosate translocation (Lorraine-Colwill *et al.*, 2002; Koger & Reddy, 2005; Powles & Yu, 2010). Increased apoplast, plastid and xylem loading was found to reduce phloem loading and therefore decrease glyphosate translocation in *C. canadensis* (Feng *et al.*, 2004). Indeed, it is now believed that the impaired translocation, observed in some populations of glyphosate resistant *C. Canadensis*, is a bi-product of the causative mechanism of resistance:

vacuole sequestration (Ge *et al.*, 2010). Mechanisms associated with impaired glyphosate translocation confer a comparatively higher resistance level than target-site modification; this may explain the high frequency of impaired translocation associated mechanisms in Australian glyphosate resistant *L. rigidum* populations (Preston & Wakelin, 2008; Preston *et al.*, 2009). The frequency of different *EPSPS* target-site mutations that endow glyphosate resistance is low compared to other herbicides, with the main mutation at the Pro-106 locus. Therefore it is probable that relatively more weed populations will evolve mechanisms associated with impaired translocation of glyphosate (Preston *et al.*, 2009; Powles & Yu, 2010).

4.1.2.3 Metabolism

It is widely established within the literature that glyphosate cannot be significantly metabolised by plants (Powles & Yu, 2010; Duke, 2011). Therefore it is unlikely that glyphosate resistance may be caused by metabolism. Many legumes metabolise glyphosate to produce the glyphosate metabolite aminomethylphosphonic acid (AMPA). However, there is no correlation between the level of glyphosate resistance and metabolite production (Reddy *et al.*, 2008). Conversely, a population of *D. insularis* (Sourgrass) appeared to have the capacity to metabolise glyphosate. Resistant plants were found to degrade >90% of glyphosate into the metabolites: aminomethylphosphonic acid (AMPA), glyoxylate, and sarcosine, compared to 11% in susceptible individuals (168 hours after treatment), (de Carvalho *et al.*, 2012). Further research is required to determine the plant enzymes responsible for the glyphosate degradation. A GOX-like enzyme is hypothesised to occur in some weed species however there has been no successful characterisation of the causative gene

or enzyme (Duke, 2011). The characterisation of the glyphosate degradation gene(s) in *D. insularis* will validate the mechanism and lead to the re-evaluation of the established doctrine that glyphosate metabolism is not a substantial mechanism of resistance.

4.1.3 Combinations of mechanisms

Multiple mechanisms of resistance may be present within a resistant population and even within a single individual. Therefore it is necessary to investigate all potential mechanisms of resistance.

Investigation into the above mentioned *A. palmeri* population from Georgia identified plants which contain the same gene copy number and had discrete levels of resistance. Therefore the Georgia population may contain a secondary mechanism of resistance. All *EPSPS* gene copies were found to be glyphosate sensitive. Therefore, it has been hypothesized that the additional variation in resistance levels is due to disparity in the presence of glyphosate exclusion mechanisms (Sammons, Unpublished data). This study indicates the potential complexity which may underlie an incidence of resistance.

Both target-site mechanisms and mechanisms associated with impaired translocation are encoded for by single nuclear genes, hence the two can be combined through cross pollination (Powles & Preston, 2006). It is believed that the outcrossing nature of *A. tuberculatus* can facilitate the rapid accumulation of several resistance mechanisms into a single population (Trucco *et al.*, 2009). In fact, an *EPSPS* target-site mutation and an impaired translocation associated mechanism were found to co-

occur, in a glyphosate resistant population of *A. tuberculatus* from Mississippi (Nandula *et al.*, 2013). These two mechanisms have also been established in a glyphosate resistant *L. rigidum* population from South Africa (Yu *et al.*, 2007). Notably four mechanisms of glyphosate resistance were found in a *D. insularis* population*;* target-site alteration, impaired translocation, reduced uptake and metabolism. However, when multiple resistance mechanisms are present within a single population, it is difficult to disentangle the contribution of each mechanism had on glyphosate resistance.

4.1.4 Objectives

- Determine the glyphosate resistance mechanism in the Renville and Dumont *A. tuberculatus* populations.
- Sequence the *EPSPS* target-site gene from resistant and susceptible *A. tuberculatus* plants from Renville and Dumont populations with a view to identifying target-site alterations that could be associated with glyphosate resistance.
- Quantify total herbicide uptake and translocation in resistant and susceptible *A. tuberculatus* plants from the Renville population.
- Determine the relative *EPSPS* target-site gene copy number in resistant and susceptible *A. tuberculatus* plants from the Renville population.

4.2 Methods

4.2.1 Target-site resistance

4.2.1.1 Target-site alteration - EPSPS sequencing

4.2.1.1.1 Plant material

EPSPS sequencing was conducted to determine whether a point mutation, insertions or deletions has occurred within the *EPSPS* gene of the glyphosate resistant phenotype. Leaf material was selected from Dumont and Renville plants and stored at -80 ^{o}C until use. A total of 3 sensitive and 5 resistant plants were sequenced. Plant material was sourced from parental plants selected through section 3.2.3.

4.2.1.1.2 RNA extraction

Approximately 100 mg of leaf tissue was frozen with liquid nitrogen and ground in a pestle and mortar. The resulting dry powder was transferred into a 2 ml Eppendorf tube and 500 µl of RNazol ® RT (Sigma-Aldrich) was added to the sample. Samples were mixed with a pipette and then centrifuged at 13000 rpm for 5 min at 4 ^{o}C. The aqueous layer was retained and 200 µl of nuclease free water was added. Tubes were shaken for 15 sec, left at room temperature for 15 min and centrifuged at 12000 rpm for 15 min. The supernatant was retained and polysaccharide contaminants were removed through the addition of 125 µl of isopropanol and 125 µl of salt solution (NaCl + NaCitrate). Tubes were shaken vigorously for 15 sec, left at room temperature for 15 min and then centrifuged at 12000 rpm for 15 min. The supernatant was discarded and an RNA-wash was conducted through the addition of

200 µl of 75 % ethanol. Tubes were centrifuged at 4000 rpm for 3 min, and the supernatant was discarded. The RNA wash step was repeated. In the second RNA wash, great care was taken to remove all of the supernatant with a P200 pipette and tubes were centrifuged for 20 sec. Any excess liquid was removed with a P10 pipette. Tubes were left to dry for 10 min in a fume hood before pellets were suspended in 100 µl of water.

4.2.1.1.3 Quantification and purification

The level of RNA and the level of contaminants present within the samples were estimated using a NanoDrop® (Labtech). If samples were contaminated with polysaccharides, the contaminants were removed through an extra RNA-wash step. For this step, nuclease free water was added to the samples until the RNA solution volume equaled 100 µl. Subsequently, additions of 25 µl of isopropanol and 25 µl of salt solution were added to the samples. Samples were shaken vigorously for 15 seconds and left to stand at room temperature for 10 minutes. The RNA washes and pellet suspensions were conducted as outlined in the RNA extraction procedure above.

4.2.1.1.4 cDNA synthesis

First strand cDNA synthesis and amplification of target cDNA was conducted using the SuperScript® III First-Strand Synthesis System for RT-PCR kit (Invitrogen™). Oligo$(dT)_{20}$ primers were used for the first strand C-DNA synthesis. Amplification of the target cDNA was conducted using three sets of primers. These primers span the entire *EPSPS* sequence and are outlined in table 4.2. Ready-to-Go Taq Beads (Amersham Biosciences) were each combined with 1 µl of DNA, 2 µl of primer mix

and 22 µl of nuclease free water to produce a reaction volume of 24 µl. PCR cycle conditions consisted of 1 cycle of 95 ^{o}C for 5 min, 45 cycles of 95 ^{o}C for 15 seconds, 60 ^{o}C for 1 min and 72 ^{o}C for 1 min, followed by 1 cycle of 72 ^{o}C for 10 min.

Table 4.2: Primer sequences used for cDNA target sequence amplification, primer sequence source: (Gaines *et al.*, 2010). Collectively these primers span the entire *EPSPS* nucleotide sequence.

Primer name	Sequence	Amplicon size
*EPSPS*_R1	5'-GTCATAAGTTCAATGGCGGTGG-3'	
*EPSPS*_F1	5'-ATGTTGGACGCTCTCAGAACTCTTGGT-3'	994 bp
*EPSPS*_R5	5'-TCTTTACCAACAGGAAACAGACCACCA-3'	
*EPSPS*_F5	5'-GCCAAGAACACAAAGCGAAATTCAGAG-3'	474 bp
*EPSPS*_R6	5'-CTATTAGTCTCAAATCAAAACTTCGGCG-3'	
*EPSPS*_F6	5'-CAGGGAATCATCTGGAAGGAAACATTTG-3'	407 bp

Gel electrophoresis was conducted on the cDNA PCR products to confirm successful amplification. Products were run against 10 µl of a 1kb ladder, on 2% agarose gel containing 0.5 µl mg^{-1} ethidium bromide, gels were run in a solution of TBE X1 buffer (pH 8.0; Tris-Base (0.089M), EDTA (0.02M) & Boric acid (0.089M)). Subsequently, 5 µl of PCR products were mixed with 5 µl of loading dye and the resulting mixture was loaded into the wells. The gel was run at 100 volts for 30 min. The extent of the DNA migration through the gel was visualised using a UV transilluminator, allowing confirmation that the desired products had been amplified (figure 4.1 a & c). The *EPSPS*_R1 and *EPSPS*_F1 primers were used for sequencing; the resulting amplicon encompasses 67% of the gene sequence and includes the common 106 codon position. DNA contaminants were removed from the cDNA

samples using the Turbo DNA-free DNAse (Invitrogen™) protocol. The DNA template was amplified through PCR with Pfu DNA polymerase enzyme to produce blunt ended PCR products for plasmid ligation (for full protocol see the Zero Blunt® PCR Cloning kit manual (Invitrogen™)). The desired sequence was ligated into a plasmid vector PCR®-Blunt (figure 4.2) using the Zero Blunt® PCR Cloning kit (Invitrogen™).

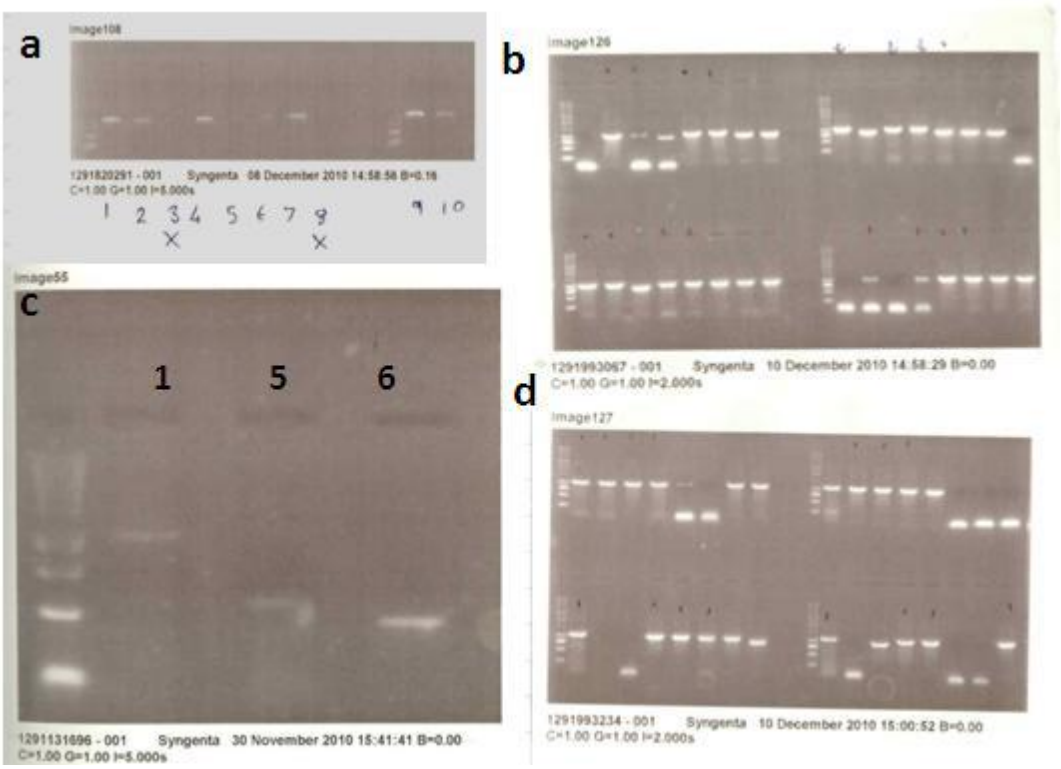


Figure 4.1: Gel Electrophesis images a. successful reverse transcription PCR's, primer set 1. Renville resistant (1, 4 & 10), Renville susceptible (2 & 7), Dumont resistant (5 & 6) Dumont susceptible (9). **b & d** Successful ligation and vector uptake after cloning **c.** Confirmation of a working reverse transcription PCR method for the *EPSPS* primer sets 1, 5, 6 from left to right. The ladder was 1kb.

Figure 4.2: The pCR®- Blunt plasmid. The kanamycin and Zeocin™ resistance genes allow the selection of *E. coli* containing the plasmid. The pUC origin allows high replication and maintenance of the plasmid within in *E. coli*. The *lac*Z α-ccdB gene leads to cell death through the inhibition of DNA gyrase. When a blunt DNA sequence is ligated between the promoter P_{lac} and the *lac*Z α-ccdB, the expression of the lethal ccdB gene is interrupted. Therefore, only *E. coli* cells containing a plasmid with a blunt DNA sequence insert will survive. Figure source: The Zero Blunt® PCR Cloning kit (Invitrogen™).

4.2.1.1.5 Cloning

PCR products were cloned to bulk the DNA and conduct high quality sequencing of the EPSPS amplicons, 2 µl of the ligated plasmids were transformed into 25 µl NEB 5- alpha competent *E. coli* cells (New England BioLabs® Inc.). The cells were incubated on ice for 30 minutes, heat-shocked at 42 ^{o}C for 45 seconds and then returned to the ice for 2 minutes. 250 µl of SOC media was added to the transformed

cells and tubes were shaken at 200 rpm in a 37 ^{o}C incubator for 1 hour. The transformed solution was pipetted onto kanamycin selection plates, in volumes of 20 and 100 µl to ensure the production of discrete colonies for selection. Any colonies which were able to grow on the kanamycin plates were seen to contain a plasmid with a sequence inserted between the P_{lac} promoter and the *lacZα ccdB* gene. Discrete colonies were selected, preserved on a master plate and screened using PCR to determine whether a single copy of the *EPSPS* F1- R1 amplicon had been incorporated into the plasmid. For PCR protocol see Zero blunt PCR cloning kit manual p12 (Invitrogen™). Gel electrophoresis was used to distinguish the colonies which contained a 1 kb DNA insert. Subsequently, colonies confirmed to contain a 1 kb DNA insert were selected for sequencing (figure 4.1 b & d). The colonies containing the 1 kb inserts were transferred into tubes containing 175 ml of Luria-Bertani broth (1% Tryptone, 0.5% Yeast Extract, 1% NaCl (pH 7)) and 175 µl of kanamycin. Tubes were incubated at 37 ^{o}C for 23 hr at 260 rpm to provide optimum conditions for bacterial growth. Purification of plasmid DNA was achieved using the Zyppy^{TM} Plasmid Mini Prep kit (Zymo Research). The resulting plasmid DNA was sequenced with the M13F & M13R oligos primers (for primer sequences see the Zero Blunt® PCR Cloning kit manual (Invitrogen™)).

4.2.1.2 Target gene amplification

4.2.1.2.1 Plant material

Leaf material was taken from F2 plants which had survived to reproduction after glyphosate application (840 g ai ha^{-1}). The F1 parental line had survived glyphosate application at 630 g ai ha^{-1}. Susceptible plant material was taken from among the F2

plants that had shown symptoms of glyphosate susceptibility at 157.5 g ai ha^{-1}. The F1 parental line had exhibited vegetative clone death at 420 g ai ha^{-1}. All plant material was frozen with liquid nitrogen and stored at -80 ^{o}C until use.

4.2.1.2.2 DNA extraction

Approximately 0.5 g of leaf material was ground in a pestle and mortar with 3 ml of grinding buffer (100mM NaOAc pH 4.8; 50 mM EDTA pH8; 500 mM NaCl; 2% PVP; 1.4% SDS; H_2O) to lyse plant cells. The sample was transferred into multiple 1.5 ml Eppendorf tubes and incubated at 65 ^{o}C for 15 min. Subsequently, 1 ml of ammonium acetate was added; tubes were mixed by multiple inversions and incubated at 65 ^{o}C for 10 min. Sample tubes were centrifuged at 13000 rpm for 10 min (RT) to precipitate the proteins. The protein free supernatant was then transferred into a new tube. A 0.6 volume of cold isopropanol was added and tubes were inverted several times. Vials were placed at -20 ^{o}C for 30 min and centrifuged at 13000 rpm for 10 min. This step allows the precipitation and isolation of DNA. The supernatant was carefully discarded to obtain the pellet of DNA which was re-suspended in 500 µl of TE. The removal of polysaccharide contaminants was achieved using 500 µl of phenol: chloroform: iso-amyl alcohol (25:24:1) (pH 8). Tubes were shaken for 1 min, stood at RT for 5 min and centrifuged at 13000 rpm for 5 min. The aqueous layer was transferred into a new tube and this phenol: chloroform: iso-amyl alcohol step was repeated twice. One volume of chloroform: iso-amyl alcohol (24:1) was added to tubes and mixed by multiple inversions. Samples were stood at RT for 5 min, centrifuged at 13000 rpm for 5 min and then the aqueous layer was transferred into a new tube. Finally the re-precipitation of

DNA was achieved through the addition of 0.1 volume of 3M NaOAc and 1 volume of cold isopropanol. Sample tubes were mixed by inversions, centrifuged at 13000 rpm for 10 min and the supernatant was discarded. Ethanol (500 µl of 70%) was added, samples were then placed in a water bath at 20 ^{o}C for 10 min, centrifuged at 13000 rpm for 5 min and the resulting supernatant was discarded. Pellets were allowed to dry for 10 min and DNA was taken up in 50 µl of water. The DNA quantity (ng μl^{-1}) and quality was assessed using a NanoDrop® (Labtech). The level of protein contamination was measured by the ratio between the absorbance at the wave lengths of 260 to 280 nm and 260 and 230 nm for protein and carbohydrate contamination, respectively. None of the samples contained protein contamination. However, many samples contained a high carbohydrate contaminant levels (< 1.7 abs at 260:230 nm). The contaminated samples were re-cleaned to remove the polysaccharides as outlined in the following procedure. NaOAC (0.1 volume) was added to the DNA suspension alongside 2.5 volume of 100% ethanol. Sample tubes were mixed by multiple inversions and placed at -20 ^{o}C for 12 hours. The samples were centrifuged at 13000 rpm for 10 min and the supernatant was discarded. Finally 500 µl of 70% ethanol was added to the pellets and samples were centrifuged at 13000 rpm for 10 min. Again the supernatant was discarded and pellets were left to dry for 10 min before re-suspension in nuclease free water.

4.2.2.1.3 Quantitative polymerase chain reaction procedure

The real time quantitative PCR procedure is used to determine the relative copy number of the gene of interest (GOI) when compared to a control (CON) gene. The core reagent, SYBR green dye, binds to double stranded DNA and produces

florescence; the level of florescence is detected at each cycle by the Q-PCR machine. The Q-PCR procedure provides a cycle threshold value indicating the number of cycles required before a critical level of amplification (or florescence) is achieved before detection. The higher the cycle threshold value, the more PCR cycles required to reach the threshold detection level and the lower the original gene copy number.

To conduct the Q-PCR, two master mixes were set up for the CON and GOI genes, each containing a ratio of 10:7:1:1 MESA Blue qPCR MasterMix Plus for SYBR® Assay mix (Eurogentec): Water: Forward primer: Reverse primer. Mesa blue master mix was pipetted last and mixed until the high viscosity disperses. Primers were diluted from the 200 µM stock to 2.5 µM. The *ALS* and *EPSPS* forward and reverse primer sequences are provided in table 4.3.

Table 4.3: Primer sequences used for quantitative PCR Primer source: Gaines *et al*., (2010) An extension of the *ALS* primer was conducted, shown in bold. This extension optimised primer binding. Additionally the underlined base was exchanged from a G to a C.

Primer name	Sequence	Amplicon length
EPSF1	5'- ATGTTGGACGCTCTCAGAACTCTTGGT- 3'	
EPSR8	5'- TGAATTTCCTCCAGCAACGGCAA-3'	195 bp
ALS_F3	5'- CGCTGCTGCTC<u>A</u>AGGCTACGCT**CG**-3'	
ALS_R3	5'- GCGGGACTGAGTCAAGAAGTG**CATC**-3'	118 bp

The treatment solution was developed using a ratio of 19:1 master mix: DNA template. After the master mix and DNA were combined, samples were vortexed at a

low speed. 15 µl of each of the resulting treatment mixtures was pipetted into three wells to produce three technical replicates within the optical plate. The optical plate was covered with optical film and sealed with an adhesive film applicator and centrifuged at 1200 rpm for 1 min at 4 ^{o}C. The Q-PCR was run on the MyiQ detection system (Bio-Rad). Q- PCR cycle conditions consisted of 1 cycle of 95 ^{o}C for 5 min, 45 cycles of 95 ^{o}C for 15 seconds, 60 ^{o}C for 1 min and 95 ^{o}C for 1 min, 1 cycle of 55 ^{o}C for 1 min. The quantification sequence was followed by an increase from 55 to 95 ^{o}C over 10 sec in order to determine the melting curve of the amplified products.

4.2.2.1.4 Primer curves

The primer efficiency curve was calculated to establish the optimum primer concentration for Q-PCR reactions. The optimum primer concentration was determined to be 2.5 µM to give a final concentration of 0.125 µM in a 10 µl reaction. The primer melting curve was checked for efficiency with a desirable slope approximating -3.14 (± 0.24). The primer curve determined the relative efficacy of the primers (between 5-10% difference in efficacy is acceptable). The DNA template strength for the Q-PCR was also determined through the primer curve: at high DNA concentrations there may be too many inhibitors present for the reaction to proceed.

4.2.2 Non target-site resistance

4.2.2.1 Absorption and transport of glyphosate

4.2.2.1.1 Plant material

Plants from seed family 153 (S) and 315 (R) (3.2.3) were sown on to levelled seed trays containing fine grade sphagnum moss peat: sand; 24:1, (pH to 5.5 – 6.0; N = 150, P = 200, K = 200 mg litre^{-1}) (FS2) soil and covered with 5 mm of growth medium. After 8 days, individual seedlings were transplanted into 7 cm biodegradable pots using a 1:1 ratio of peat to compost and moved to the growth chamber for acclimatisation. Growth chamber conditions were 24 ^{o}C: 18 ^{o}C (day: night), 16:8 hr, 70%. Humidity and lighting were provided for 16 hr at an intensity of 800 µmol m^{-2}s^{-1}. Plants were watered on requirement.

4.2.2.1.2 ^{14}C glyphosate tracing

After 14 days, uniform plants were selected for the experiment. The third fully opened leaf was selected as the treatment leaf and a 2 cm wide strip was marked to provide a guide for droplet application.

Experiment one investigates the discrepancy in uptake between R and S plants. Six replicate plants were used for each of the eight treatments. The treatments consisted of four time points and two phenotypes to produce a total of 48 experimental plants. Plants were assessed at 0, 6, 24 and 72 hours after treatment and combustion analysis

was used to assess data (section 4.2.2.3). An average of 5248 Bq ^{14}C glyphosate was applied to each plant.

Experiment two investigates the discrepancy in impaired translocation between R and S plants. 12 replicate plants were used for each of the eight treatments. The treatments consisted of four time points and two phenotypes to produce a total of 96 experimental plants. Plants were assessed at 0, 6, 24 and 48 hours after treatment and Aida image analysis (Version 3.20.007, Raytest Isotopenmessgerate GmbH, Straubenhardt, Germany) was used to assess data. An average of 5517 Bq ^{14}C glyphosate was applied to each plant.

4.2.2.2 Application of glyphosate

The glyphosate treatment solution contained Touchdown IQ 360 and was applied at a rate of 840 g ai ha^{-1} in an equivalent spray volume of 190 g l ha^{-1}. Therefore each plant was treated with a total glyphosate concentration of 4.42 mg ml^{-1}. In order to produce a solution of the desired concentration and radiation level, a proportion of the glyphosate volume was replaced by the ^{14}C labelled glyphosate stock solution. The volume was calculated to produce a radiation level of 5000 Bq per 4 µl of glyphosate solution.

A total of 4 µl of ^{14}C labeled glyphosate solution was applied to laminar sections of the treatment leaf as 20 X 0.2 µl droplets using a Hamilton syringe. A total of 17.68 µg of glyphosate was applied to each plant. Experimental plants were arranged in a randomised block design.

A radiation base level was determined using the treatment solution; six replicate control vials were produced before and after treatment application. Vials contained 20 X 0.2 µl of the glyphosate solution droplets, applied to glass cover slips. Quantification of the radiation level was achieved through the addition of scintillation fluid to the vials and the level of decays per second was measured as outlined for the foliar surface residue (below). No change in the ^{14}C radioactivity of the glyphosate solution was observed from the beginning to the end of the treatment application process, in either experiment. Therefore, no evaporation of the treatment solution occurred and the proportion of radiation applied during treatment application is consistent.

4.2.2.3 Harvesting

Before harvesting, at each time point, the excess foliar surface residue was recovered from the treated leaf using 3 X 1 ml washes of 50:40:10 Water: Acetonitrile: THF solvent. Subsequently, 10 ml of scintillation fluid was added to the residue vials and the ^{14}C radiation was measured using a liquid scintillation counter (Perkin Elmer Tri-carb 2900TR). The residue was taken from the zero time point (T0) to provide a radiation control comparison for later time points.

For each harvest, plants were lifted out of the pots, and soil was gently shaken and washed from roots in a beaker of water, whole plants were laid between sheets of blotting paper, so that all individual leaves could be distinguished. The pressed plants were placed at -20 ^{o}C for 2 days before freeze drying in a vacuum freeze drier (Edwards Modulo) for 24 hours. The samples were subsequently spray mounted onto card before covering with a layer of mylar film, before image processing. This card

was placed, face up, in a Fujifilm cassette 2040 and a phosphor-image plate (Fujifilm BAS-MC, 2040 20-40 cm) was inserted on top. All phosphor-image plates were blanked before use with a Raytex light box image-plate-eraser III. Cassettes were left for 24 hr to allow images to develop. The plates were scanned using the phosphorimager (Fuji FLA5000) to produce images.

The level of radiation was quantified for plants from experiment one, through combustion analysis. Plants were divided using a scalpel into 6 sections: meristem, treated area, above treated area, roots, stem and the rest of the foliage to produce a grand total of 288 sections. Sections were weighed, wrapped in 4 X 4 cm squares of Kimwipes® tissue paper and placed in ceramic boats. The ceramic boats were run through the Zinsser Robox 192 Biological oxidiser with OX500 (Harvey) which combusts samples and traps the evolved $^{14}CO_2$ in vials through Oxysolve C400 (3-methoxyproylamine and 1, 2, 4 trimethyl benzene solvents). Scintillation fluid (10 ml) was added to each of the combustion vials and analysed as indicated above for the foliar wash vials. Positive and negative control samples were combusted at regular intervals throughout the oxidising process to check the efficacy throughout measurements. As this was a lengthy process, in experiment 2, image radiation quantification was assessed using Aida (Image analysis software), where plant segments were selected to determine the level of radiation contained within each segment: treated leaf, the rest of the foliage, stem, and roots. No further processing was undertaken of experiment 2 plants.

4.2.3 Data analysis

4.2.3.1 Sequence analysis

DNASTAR was used to conduct sequence analysis of the *EPSPS* locus. SeqMan Pro was used to align forward and reverse reads and assemble a consensus sequence. Primer sequences were identified at the ends of the consensus sequences and removed. The clustalW algorithm in Meg align was used to align sequences against an EPSPS sensitive sequence downloaded from Genbank, (Accession: FJ869881.1; GI: 257792899 up loaded by Gaines et al., 2010). Subsequently, the DNA sequences were analysed for point mutations, insertions or deletions.

4.2.3.2 Q-PCR, Uptake and Translocation analysis

The Bartlett's test for homogeneity and the Shapiro-Wilk test for normality were used to confirm that the data conforms to the assumptions of a parametric analysis. If the distribution deviates from a normal distribution or there was a difference in variance of the treatments, then the data was transformed using square root or log functions. The response variables were relative gene copy number, total glyphosate in plants and phloem translocated glyphosate and each comparison was made between resistant and susceptible plants. Any data which conformed to these assumptions was analysed using a one or two way ANOVA. Significant differences between treatments were determined from the two way ANOVA analysis by comparing the treatment means with the LSD. Any data which could not be transformed to conform to the assumptions was analysed using a Mann-Whitney U test.

4.3 Results

4.3.1 Target-site resistance

4.3.1.1 EPSPS Target-site Sequencing

Sequence analysis of the *EPSPS* cDNA showed no point mutation in the 994 bp amplicon section of the *EPSPS* gene that correlated with the resistant phenotype. The five resistant and three susceptible plants were selected from across the Dumont and Renville populations. Four amino acid sequence alterations at the Pro-106 locus are known to endow glyphosate resistance (table 4.1); therefore this locus was investigated with particular interest. However, no such mutation was observed. Additionally, the amino acid sequence alterations at Gly-101, Thr-102, Pro-182 & Tyr-310 positions, reported to confer glyphosate resistance, were not observed (Eschenburg *et al.,* 2002; Funke *et al.,* 2009; Powles & Yu, 2010; de Carvalho *et al.,* 2012). Table 4.4 shows the *EPSPS* DNA sequence corresponding to the 101- 106 amino acids.

Table 4.4: 300-318 bp *EPSPS* DNA consensus sequences for 5 resistant and 3 susceptible *A. tuberculatus* plants. The 300-318 bp region is the common site of resistance endowing mutations. This sequence region translates to the amino acid sequence: GTAMRP from position 101 to 106. The underlined base indicates a SNP. Ref. is the wild type reference sequence, EPSPS sensitive sequence downloaded from Genbank, (Accession: FJ869881.1; GI: 257792899 up loaded by Gaines et al., 2010). Plant number corresponds to seed family number in 3.2.3.

Plant number	Population	Phenotype	EPSPS DNA Sequence 300-318 bp
Ref.	-	Susceptible	GGAGGATGATAATACAGA
315	Renville	Resistant	GGAGGATGATAATACAGA
253	Renville	Susceptible	GGAGGATGATAATACAG<u>C</u>
1	Renville	Resistant	GGAGGATGATAATACAGA
29	Dumont	Resistant	GGAGGATGATAATACAGA
277	Dumont	Resistant	GGAGGATGATAATACAGA
96	Renville	Susceptible	GGAGGATGATAATACAGA
77	Dumont	Susceptible	GGAGGATGATAATACAGA
226	Renville	Resistant	GGAGGATGATAATACAGA

As mentioned above, there was no resistance endowing mutation at the Pro-106 locus in the *EPSPS* gene observed in the sampled plants; however, a single nucleotide polymorphism (SNP) was found in the last codon base (adenine to guanine) of plant 253 (Table 4.4). The SNP was silent and therefore does not result in an amino acid substitution. There was an amino acid substitution resulting in an

Ala-71- Asp alteration, however, this is not a known resistance endowing mutation, additionally the mutation does not segregate amongst resistant individuals.

4.3.1.2 Target Gene amplification

Gene copy number was quantified to determine the potential for an *EPSPS* gene amplification mechanism of glyphosate resistance. The relative gene copy numbers for each plant are in figure 4.3. The average R relative gene copy number was 14.1 (± 4.88) and the average S relative gene copy number was 0.99 (± 0.04). When samples were grouped by phenotype and analysed using a Mann-Whitney U test there was a significant difference in gene copy number between phenotypes ($W= 28$; $P< 0.05$). However, it is clear from figure 4.3 that the plants do not represent two distinct genotypes (R and S) but a continuum of gene copy numbers. Notably, the resistant individual R2 has survived to reproduction after the application of 840 g ai ha^{-1} glyphosate and the parental lines have survived 630 g ai ha^{-1}. However, this individual does not appear to have an amplified *EPSPS* gene copy number (figure 4.3). The resistant individual R2 has either received a suboptimal dose of glyphosate or the plant has survived glyphosate application due to a secondary resistance mechanism. This potential is discussed further in section 4.4.

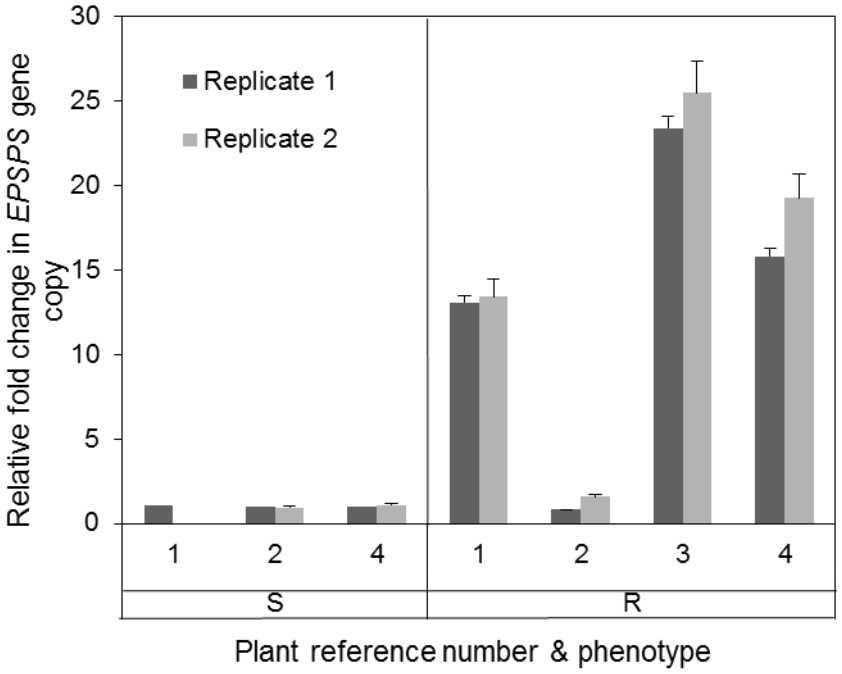


Figure 4.3: The relative fold change in *EPSPS* gene copy number when compared to the *ALS* gene copy in three susceptible and four resistant *A. tuberculatus* plants from the Renville population.

4.3.2 Non target-site resistance

4.3.2.1 Absorption of glyphosate

There was no significant difference in the uptake of glyphosate between resistant and susceptible plants at each of the time points (figure 4.4). The total ^{14}C glyphosate uptake of the resistant and susceptible plants is in figure 4.4. This finding indicates that the impairment of glyphosate uptake was not a mechanism of glyphosate resistance within the Renville population. The majority of glyphosate uptake occurs before the 6 hour time point.

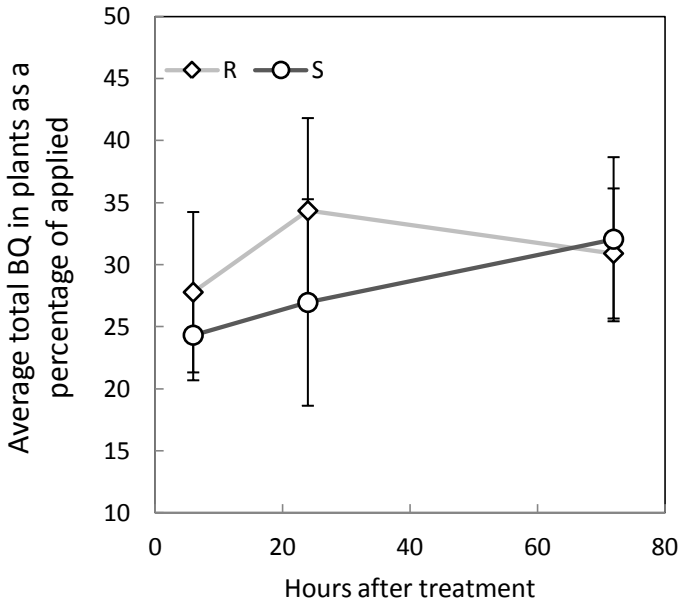


Figure 4.4: The average uptake of ^{14}C glyphosate at 6, 24 and 72 hours after treatment within the susceptible and resistant plants. The ^{14}C glyphosate levels were taken as a percentage of the ^{14}C glyphosate recovered from the leaf surface at 0 hours after treatment. Error bars are standard error of the mean.

4.3.2.2 Translocation of glyphosate

The initial experiment found there was no significant decrease in glyphosate translocation or the level of glyphosate found in the meristematic growing tissue in resistant compared to susceptible plants, at all time points (figure 4.6). These findings suggest that no impaired translocation of glyphosate is present within the Renville population and therefore such associated mechanisms do not contribute substantially, to glyphosate resistance. There is no significant difference in the dry biomass or the glyphosate concentration (total µg of glyphosate/ dry biomass) of the resistant and susceptible plants at any of the time points. Also there was no

significant difference between the glyphosate concentrations of the treated leaf or the total plant minus the treated leaf for the susceptible and resistant plants, at any of the time points (not shown).

The total ^{14}C glyphosate phloem translocation was measured by the intensity of luminescence in the non-treated plant regions as a proportion of the total area selected for analysis divided by the total luminescence present in the whole plant. There was no significant difference between the total level of phloem translocation (all plant minus the treated leaf) as a percentage of the total glyphosate uptake in resistant and susceptible plants at 6, 24 and 48 hours after treatment (figure 4.5). Additionally, there was no significant difference across the time points, indicating the proportion of glyphosate translocation in resistant and susceptible plants does not alter over time (figure 4.5). Therefore impaired glyphosate translocation associated mechanisms were not a major contributor to the resistance in the Renville *A. tuberculatus* population.

Further to this analysis it is worth noting that there was no significant difference between the total area of the image selected for quantification in all time point and phenotype treatments. Additionally, when the luminescence data was analysed without adjusting for the image area, there was no impact on the final conclusions for impaired translocation (analysis not shown).

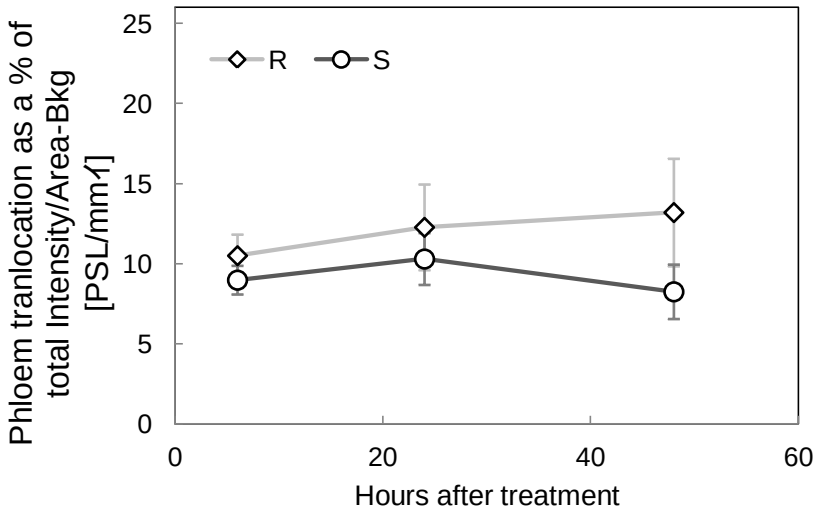


Figure 4.5: The level of ^{14}C glyphosate phloem translocation as a percentage of the total luminescence, as measured by the intensity of the Photo-stimulated luminescence as a proportion of the plant area minus the background luminescence, in resistant and susceptible phenotypes at three time points. Error bars are standard errors of the mean.

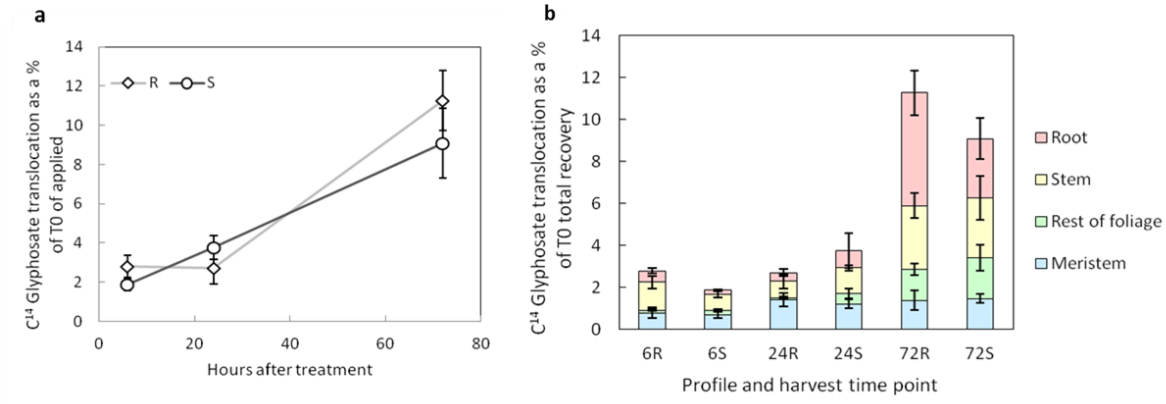


Figure 4.6: The level of ^{14}C glyphosate phloem translocation found in the combustion analysis a. The average distribution of translocated C^{14} glyphosate within the susceptible and resistant plants at 6, 24 and 72 hours after treatment. The C^{14} glyphosate levels are taken as a percentage of the C^{14} glyphosate recovered at 0 hours after treatment. **b.** The proportion of C^{14} glyphosate translocated to region below the treated area.

4.4 Discussion

No target-site point mutation was found at the P-106 *EPSPS* codon position in the resistant individuals from the Dumont and Renville populations. Similarly, there was no conclusive evidence for the impaired translocation or uptake of glyphosate, in the resistant individuals selected from the Renville population. However, three out of four resistant plants exhibited *EPSPS* gene amplification.

4.4.1 Target gene amplification

The evolution of the *EPSPS* gene amplification mechanism of glyphosate resistance had been characterised before it had evolved within an agricultural setting. Namely, lab experiments selected for *EPSPS* gene amplification in *G. max* (maize), *Medicago sativa* (alfalfa) and *Nicotiana tabacum* (tobacco) plant tissue cultures (Widholm *et al.*, 2001). *EPSPS* gene amplification resulted in a 105-420 fold increase in the culture resistance level, as indicated by the comparison of the LD_{50}'s before and after selection. These results confirm that *EPSPS* gene amplification can cause glyphosate resistance and that this resistance mechanism may evolve across a range of plant taxa in the lab (Widholm *et al.*, 2001). As mentioned in the introduction, the first reported incidence of field-evolved glyphosate resistance was mediated by *EPSPS* gene amplification found in an *A. palmeri* population from Georgia, USA (Gaines *et al.*, 2010). The *A. palmeri* plants that contained multiple *EPSPS* gene copies exhibited lower shikimate accumulation after glyphosate treatment when compared to sensitive plants (Gaines *et al.*, 2010). This finding confirms that an amplified *EPSPS* gene copy number results in glyphosate resistance. There was a directly proportional correlation between the gene copy number, level of mRNA transcript and the quantity of EPSPS in *A. palmeri* from the Georgia population (Gaines *et al.*, 2010). This relationship has not been explored in the Renville *A. tuberculatus* population however section 5.3.2 indicates a positive correlation between seed family LD_{50} and *EPSPS* relative gene copy. Therefore the gene copy number was good proxy for mRNA transcript, EPSPS protein and resistance levels. In A. palmeri, the modal copy number was 21 to 39 copies amongst freely pollinating plants (Gaines *et al.*,

2010). This copy number was of equal magnitude to that found in the Renville population of *A. tuberculatus* (section 4.3.1.2).

4.4.1.1 EPSPS expression levels

It is important to remember that the genomic analysis of copy number results in the inclusion of pseudogenes and down regulated genes. Therefore the use of DNA to determine gene copy number may result in an overestimation of the functional gene copy level. The impact of this potential issue may be mitigated by the extraction of mRNA from each plant; this information will complement the DNA data to provide an indication of whether gene copy number and expression levels correlate. Indeed, *EPSPS* gene copy number and expression levels were found to correlate, in the *A. palmeri* population from Georgia (Gaines et al. 2010). Additionally, further research is required to determine whether the *EPSPS* gene copies, identified in the experimental plants are expressed constituently or whether genes are inducible. This may be achieved by measuring the EPSPS expression levels in plants before and after glyphosate application. If the inconsistency in cDNA gene copy number is inducible, then complications may be associated with obtaining a representative EPSPS expression level for each plant. It is therefore possible that the true resistance level may not be detected without stress induction.

4.4.2 The potential origin of gene amplification

The lack of tandem repeats within the *A. palmeri EPSPS* sequence suggests that the gene amplification was unlikely to have resulted from random crossing over or rolling circle replication (Gaines *et al.*, 2010). It is hypothesized that the origin of

gene amplification within the *A. palmeri* population, was caused by a mobile genetic element associated with the *EPSPS* gene (Gaines *et al.,* 2010). One such mobile element is the transposon. Transposons are DNA sequences which are able to replicate and randomly insert themselves throughout multiple areas of an organism's genome. Indeed, the FISH mapping conducted by Gaines *et al.,* (2010) indicates that multiple gene copies have been randomly inserted throughout the chromosomes of the *A. palmeri* genome. Miniature inverted-repeat transposable elements (MITEs) were found downstream of the *EPSPS* copies (Gaines *et al.,* 2013). MITEs are small DNA sequences which can amplify a sequence within a genome from one to a thousand copies (Wessler *et al.,* 1995). The presence of these MITEs indicates a potential gene amplification mechanism. However, this sequence was found downstream of both amplified and non-amplified *EPSPS* gene copies (Gaines *et al.,* 2013). MITE sequences are highly abundant transposable elements and are often associated with multiple genes throughout a plants genome, therefore the co-existence between the *EPSPS* gene and the MITE may not be causal (Wessler *et al.,* 1995). A retro-transposon is hypothesised to have caused gene amplification (Gaines *et al*., 2013). Retro-transposons are normally inactive in plant genomes, as a result of methylation and histone binding. However, these sequences may become activated through interspecies hybridisation, abiotic or biotic stress (Kawakami *et al.,* 2010).

4.4.2.1 Introgression

Hybridisation occurs between *A. tuberculatus* and multiple *Amaranth* species including *A. hybridus*, *A. palmeri*, *A. powellii*, *A. retroflexus* and *A. spinosus*, (Murray, 1940). Controlled pollination events can result in *A. palmeri* and *A.*

tuberculatus hybridisation (Wetzel *et al.*, 1999; Franssen *et al.*, 2001; Steinau *et al.*, 2003; Trucco *et al.*, 2007). Interspecies hybridisation can result in the transfer of the *EPSPS* gene amplification trait from *A. palmeri* species to *A. tuberculatus* (Gaines *et al.*, 2012). Indeed hybridisation between *A. palmeri* and *A. tuberculatus* can occur within the field, with <0.2% hybridisation due to pollen transfer from *A. palmeri.* Further to the potential for introgression, the two species exist sympatrically (with overlapping territories) in the USA and thus, there is the potential for the introgression of beneficial adaptations to occur between species (Gaines *et al.*, 2012). However, *A. palmeri* is not found in Minnesota therefore it is unlikely that introgression has caused the *EPSPS* gene amplification mechanism in *A. tuberculatus*. As mentioned above, Gaines et al (2010) suggest that transposable elements may be responsible for the amplification of the *EPSPS* gene. Further hypothesises go on to outline that the mobilisation of transposable elements may have been induced by hybridisation. It is believed that the activation of retro-transposons may be caused by the under-methylation that occurs as the result of hybridisation (O'Neill *et al.*, 1998; Kawakami *et al.*, 2010; Gaines *et al.*, 2012).

4.4.2 Inheritance of gene copy number

The gametes of plants are established from the somatic meristematic growing cells (Wessler *et al.*, 1995). Retro- transposon amplification potential is normally silenced through methylation during the developmental stage to avoid detrimental insertion in to essential sequences (Wessler *et al.*, 1995; O'Neill *et al.*, 1998). Without the normal epigenetic silencing associated with retro-transposons, gene amplification can occur at each cell division. Thus, without such silencing, gene copy may vary

dramatically from the parent to progeny. An active transposon would cause difficulties regarding the production of experimental material. If the gene amplification within the Renville population was caused by a retro-transposon, then amplification may have occurred within somatic cells leading to the disruption of the parental and progeny gene copy number relationship. Indeed, research on the inheritance of gene copy numbers in *A. palmeri,* found that vegetative clones from the same plant, could contain different gene copy numbers to the parental plants and even between clones taken from the same plant. However, this relationship was only found in one out of four experimental seed families (Ward, Unpublished). Before any stipulations of copy number and inheritance can be made, more information is required on the mechanism of gene amplification in the *A. tuberculatus* population.

4.4.3 Mechanisms of glyphosate resistance in A. tuberculatus

Populations of *A. tuberculatus* have evolved glyphosate resistance across 13 states of the US, so far (Heap, 2013). A glyphosate resistant population of *A. tuberculatus* from Missouri, US was found to contain multiple individuals with multiple *EPSPS* gene copies; however, some of the individuals containing amplified gene copy numbers were not resistant to glyphosate. These findings indicate a complex mechanism of glyphosate resistance in the *A. tuberculatus* Missouri population (Bell *et al.*, 2009). The *EPSPS* gene copy number was used as a diagnostic tool to indicate the presence or absence of resistance in *A. tuberculatus* (Tranel *et al.*, 2011). However, the gene amplification mechanism of resistance has not been confirmed even though it is apparent that an association between gene copy number and resistance is assumed.

The Pro- 106- Ser mutation has been found in a population of glyphosate resistant *A. tuberculatus* from Illinois (Tranel et al. Unpublished). However, the resistance mechanism within this population, is believed to be complex, as even though this mutation is known to endow resistance in multiple alternate species, this mutation was found in both resistant and susceptible individuals (Tranel et al. Unpublished). There was no target-site sequence mutation within the Renville and Dumont populations.

As mentioned above a population of glyphosate resistant *A. tuberculatus* (Tall Waterhemp) from Mississippi contains two resistance endowing mechanisms: a Pro-106-Ser target-site mutation and the impairment of glyphosate translocation. Tall Waterhemp comes from the same species as the Common Waterhemp (*Amaranthus tuberculatus var. rudis*) populations from Minnesota but is a classified as a different sub-species. As Tall and Common Waterhemp are the same species they can easily breed and exchange resistance mechanisms (Nandula *et al.*, 2013).

4.4.4 Potential for multiple mechanisms

As mentioned in section 4.1.3, the glyphosate resistant population of *A. palmeri* from Georgia contains the gene amplification resistance mechanism alongside a secondary glyphosate resistance mechanism (Sammons, Unpublished data). Evidence for a secondary mechanism of resistance is in figure 4.3, where a resistant individual does not exhibit an increased *EPSPS* gene copy number. The complexity associated with isolating the effect of each mechanism in plants containing multiple resistance mechanisms, results in the potential for minor resistance mechanisms to be overlooked. The influence of the *EPSPS* gene amplification needs to be removed to

determine if a secondary mechanism of resistance may be present in the Renville population.

4.4.5 Conclusion

In conclusion, the amplification of the *EPSPS* gene is associated with glyphosate resistance in *A. tuberculatus* plants from the Renville population. Therefore, the glyphosate resistance found in the Renville population, can be attributed (in part, if not completely) to gene amplification. A secondary mechanism of resistance may be present within the Renville population due to the lack of gene amplification seen in one of the resistant individuals. The combustion analysis found no significant difference in glyphosate uptake and the combustion and image analysis found no significant difference in phloem translocation between the resistant and susceptible plants at 6, 24 and 72 hours after treatment. Lastly, there was no target-site mutation present in the Renville or Dumont resistant individuals at the Pro-106 site of the *EPSPS* gene sequence. The majority of fitness cost studies are conducted on populations with a target-site mutation mechanism of resistance (Vila- aiub et al., 2009) and, because there was no sequence alteration found within the Renville population, this study has the potential to provide a new insight into fitness costs and growth penalties associated with a polygenic mechanism of resistance, a relatively unexplored area. A recent review has speculated that work should be conducted to determine the fitness costs associated with *EPSPS* gene amplification associated with *Amaranths* (Darmency, 2013).

5.0 Assessing resistance trade-offs in *Amaranthus tuberculatus* plants in the absence of competition

5.1 Introduction

5.1.1 Single generation fitness comparison

5.1.1.1 Single populations

Herbicide resistance costs in plants are predominantly elucidated by the growth and fitness comparison of resistant (R) and susceptible (S) individuals in the absence of the selective agent (Bergelson & Purrington, 1996; Strauss & Agrawal, 1999; Vila-Aiub *et al.*, 2009a; Vila-Aiub *et al.*, 2011). Such fitness comparisons have been conducted on populations containing an agriculturally evolved resistance trait and on the model species *Arabidopsis thaliana* (Roux *et al.*, 2004).

As outlined in section 3.1 the impact of a resistance trait cannot be elucidated if a fitness study utilises discrete R and S populations due to confounding genetic variation (Purrington & Bergelson, 1997; Neve, 2007). Thus, R and S phenotypes must be segregated from a single population, by phenotyping of parental plants, to produce crosses of either; multiple parents (segregating populations) or single pair matings (segregating crosses) (Bergelson & Purrington, 1996). Comparisons using segregating crosses were found to exhibit resistance costs more frequently than segregating population comparisons, due to the lower background genetic variation

(Purrington & Bergelson, 1997; Roux *et al.*, 2004; Vila-Aiub *et al.*, 2005; Menchari *et al.*, 2008; Vila-Aiub *et al.*, 2011).

When the experimental material for a fitness cost assessment has been obtained from an agriculturally evolved herbicide resistant population, as opposed to a model organism, the resulting fitness conclusions may be directly applied to agricultural weeds and as such inform herbicide resistance management (Paris *et al.*, 2008; Neve *et al.*, 2009; Vila-Aiub *et al.*, 2011). A single generation fitness comparison method can elucidate the traits responsible for fitness costs, as opposed to a multi-generational method which predominately aims to measure a reduction in the resistance allele over time (Roux *et al.*, 2006). Such traits may be manipulated in a field situation to aid resistance control, for example: the Ile-1781–Leu ACCase resistance mutation in *L. rigidum* displays higher seed dormancy under low light levels when compared to the susceptible genotype (Vila-aiub *et al.*, 2005). Therefore, the tillage of soil may result in a lower percentage germination of resistant seed.

5.1.1.2 Multiple population fitness cost comparisons

The multiple population fitness comparison method analyses numerous R and S populations to determine the fitness cost of a resistance trait across different genetic backgrounds (Cousens *et al.*, 1997; Strauss & Agrawal, 1999; Vila-Aiub *et al.*, 2011). The resistant experimental populations used for multiple population comparisons must contain the same resistance allele mutation(s), as resistance costs are mutation specific (Roux *et al.*, 2004; Vila-Aiub *et al.*, 2009a). Unfortunately, population structure has a greater influence on fitness than resistance alleles,

therefore, multiple population comparisons may lead to the false observation or the over or underestimation of fitness costs (Lander & Schork, 1994; Vila-Aiub *et al.*, 2011).

5.1.1.3 Fitness measures for single generation studies

In single generational studies the best indicative measure of plant fitness is total viable seed production (Bergelson & Purrington, 1996; Vila-Aiub *et al.*, 2011). This measure indicates the potential resistance allele contribution to subsequent generations and thus the fitness level of a phenotype. However, ideally fitness should be measured in terms of the allele frequency over multiple generations to provide a true description of an allele's fitness (Primack & Kang, 1989; Lawrence, 2005; Vila-Aiub *et al.*, 2009a).

5.1.2 Multiple generation fitness comparisons

Multiple generational comparisons can quantify the frequency of resistance allele(s) within a population over successive generations, in the absence of the selective agent (Primack & Kang, 1989; Roux *et al.*, 2005a; Lawrence, 2005; Vila-Aiub *et al.*, 2009a). Allele frequency determination has been utilised to study herbicide resistance costs in the model species *A. thaliana* (Roux *et al.*, 2005a), however, few studies have used this method to establish the fitness of an agriculturally evolved resistance trait. For example, when glyphosate resistant *L. rigidum* populations were grown in the absence of selection, under crop competition, a substantial reduction in the resistance trait was observed over four generations (Preston *et al.*, 2009). The multiple generation method can discern strong fitness penalties; however, small

penalties may not be detected due to the potential for fixation of slightly deleterious alleles through genetic drift. In order to elucidate small fitness costs, the impact of genetic drift can be minimised through the use of large experimental populations or the use of an artificial cline on a finite population (Roux *et al.*, 2006). Artificial cline allele frequency experiments encompass a spectrum of plots, each containing an increasing R:S ratio allowing the migration of individuals between adjacent plots to mitigate the impact of genetic drift (Paris *et al.*, 2006; Roux *et al.*, 2006).

5.1.2.1 Problems of multiple generational studies

Regrettably, the multiple generational experiments require the genotyping of numerous individuals and a substantial amount of spatial and temporal investment (Roux *et al.*, 2006). Furthermore, the use of resistant experimental material in field experiments often leads to ethical concerns due the containment issues associated with the release of herbicide resistant pollen and seed into the environment (Busi *et al.*, 2008).

5.1.2.2 Fitness cost comparisons of a quantitative trait

Where resistance is caused by a monogenic resistance trait, distinct R and S seed lines may be segregated with relative ease. However, a polygenic resistance trait may require multiple rounds of segregation before distinct R and S seed lines can be produced. Indeed, a polygenic resistance trait will result in quantitative variation in resistance within populations (assuming that increasing the number of alleles proportionally enhances resistance through synergistic allele epistasis). Therefore, it would be an oversimplification to characterise seed lines into categorical

phenotypes. In this case, a series of seed families may be produced with contrasting levels of resistance to allow assessment of the correlation of resistance and fitness in the absence of the selection pressure. This method has been utilised in the study of glyphosate resistance in *Ipomoea purpurea* (L.) on half sibling seed families (Baucom & Mauricio, 2004).

The incomplete segregation of R and S seed lines (section 3.3) and the quantitative mechanism of resistance found within the Renville population (section 4.3.1.2) has enabled a comparison of resistance costs between seed families with contrasting levels of phenotypic resistance within the Renville population. Thus, Renville seed families (half siblings from a single maternal plant) containing distinct levels of resistance (initially measured by the LD_{50}) have been selected for analysis. Seed families will be used to determine whether there is a trade-off between resistance level and fitness when grown in the absence of glyphosate. Furthermore, seed family experiments can determine whether there is a positive relationship between average relative seed family *EPSPS* gene copy number and resistance (figure 5.1a). Experiments can also determine whether there is a negative relationship between seed family gene copy number and fitness in the absence of glyphosate (figure 5.1b). Finally, experiments can determine whether there is a negative association between seed family resistance level and fitness in the absence of glyphosate (figure 5.1c).

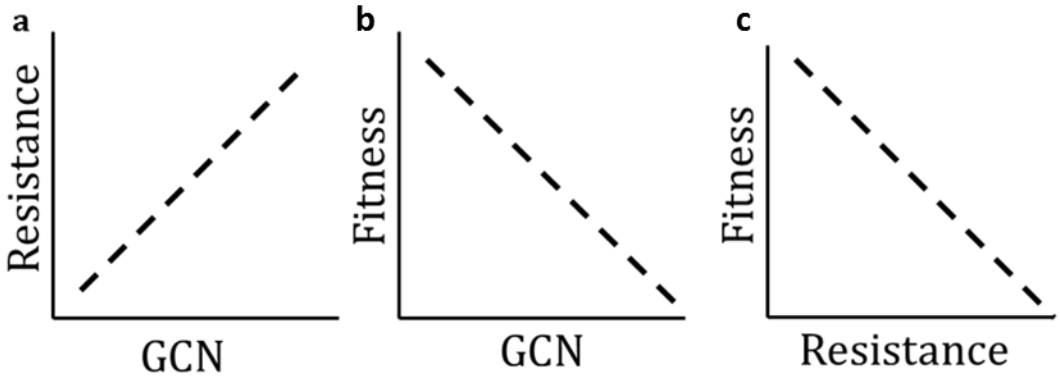


Figure 5.1: Theoretical relationships between fitness, gene copy number (GCN) and resistance in the presence of a negative trade-off. Lines represent the direction of the relationship; however, the relationship between the two variables may not be linear. **a.** A positive relationship between gene copy number and resistance level, **b.** A negative relationship between gene copy number and fitness, **c.** A negative association between resistance and fitness, which is a bi-product of the relationship between with gene copy number and resistant (a) and gene copy number and fitness (b).

5.1.3 Objective

To determine whether there is a negative trade-off between the level of glyphosate resistance (or *EPSPS* gene copy number) in *A. tuberculatus* seed families and fitness in the absence of glyphosate, under glasshouse and polytunnel growth environments.

5.2 Methods

5.2.1 Seed Family dose response

A repeat seed family dose response experiment was conducted to validate seed family LD_{50} resistance measures produced in the initial dose response (conducted on the seed families produced through the clone protocol) (section 3.3.3). The second dose response experiment was conducted in a polytunnel growth environment, this allowed the resistant indices to be correlated to those established in the glasshouse experiment and thus established whether the growth environment impacted resistance. Both dose response experiments were used to confirm that the seed families selected for the polytunnel growth and fitness cost experiments contained discrete resistance levels.

The dose response experiment was conducted on the Renville seed families selected for the growth and fitness cost experiments. Seed families were 1, 315, 90, 29, 141 and 153. Plants were produced as outlined in section 3.2.1.1, with the alterations listed below. Eight seedlings were transplanted into 1.5 L pots containing a mixture of top soil: medium grade sphagnum moss peat in a ratio 2:1 (pH =7.6, K= 176.3, P= 80.4, NO_3= 146.6, mg = 377.9 µg g^{-1}). The experiment was conducted in a polythene tunnel from June- July. Three replicate pots were produced for each of the 42 treatments consisting of six spray rates and six seed families, plus the S2 sensitive population. Pots were treated with 1680, 840, 420, 210, 105 or 0 g ai ha^{-1} of glyphosate (section 3.2.1.2). A 200 L ha^{-1} application volume was used and nozzle height was set at 50 cm above the height of the pots. A randomized block design

compensated for any discrepancies in environmental conditions (figure 5.2) Plants were assessed as outlined in section 3.2.1.3.

Figure 5.2: Photo of second seed family dose response experiment 14 days after glyphosate application. The three replicate blocks contain a replicate from each treatment, blocks were 4 rows of pots; the vertical aisle allows watering of central pots.

5.2.2 EPSPS gene copy number determination

5.2.2.1 Seed material

Eight plants were selected from each seed family (1, 29, 90, 141, 153, and 315) for *EPSPS* gene copy number quantification. Plants were selected for analysis from harvest three of the polytunnel growth and fitness experimental plants (section 5.2.3.2). Eight plants from each of the six seed families were selected for analysis. Selection of plant material occurred after the harvest, thus selection could be based upon plant biomass. Plant above ground dry biomass data was ordered from lowest to highest for each seed families, the data was divided into four equal cohorts and two plants were randomly selected from each cohort. This plant biomass selection process allowed plants representing a spectrum of sizes to be randomly selected from each seed family for gene copy number assessment.

5.2.2.2 Quantitative PCR

DNA extraction (section 4.2.1.2.2) was conducted on plants selected for analysis. Q-PCR was conducted using two Q-PCR machines; the LightCycler®480 (Roche) and the MyiQ detection system (Bio-Rad). The protocol was conducted as outlined in section 4.2.2.1.3 with the exception of the following deviations. For the LightCycler®480 (Roche) Q-PCR protocol, 10 µl of reaction solution was used per well, four replicate wells were produced per treatment and the reaction solution contained a 5:4:1:1:2 ratio of MESA Blue qPCR Master Mix Plus for SYBR® Assay mix (Eurogentec): Water: forward primer: reverse primer: DNA. Two to five Q-PCRs were conducted for each sample plant on different optical plates depending on the consistency of the Q-PCR results. The mixed model (section 5.2.4.2) standardised the resulting gene copy number values for variation between replicate wells, replicate optical plates, Q-PCR protocols and Q-PCR machines. A total of 43 plants were successfully genotyped to allow an average *EPSPS* gene copy number to be calculated for each seed family.

5.2.3 Resistance costs in the absence of selection

5.2.3.1 Contrasting growth environments

Two studies were conducted to explore the impact of environmental conditions and growth medium on growth and fitness in seed families with different resistance levels. The growth and fitness cost studies were conducted in the absence of glyphosate application in either a polytunnel (2011) or a glasshouse (2012). The polytunnel contained no supplementary heating or lighting and plants were grown in

a high nutrient growth medium (N = 800, P= 600, K=800 µg g^{-1}). However, the glasshouse, contained supplementary lighting and heating, and plants were grown in a lower nutrient growth medium (NO_3= 146.6, P= 80.4, K= 176.3 µg g^{-1}).

5.2.3.2 Polytunnel Growth and Fitness Experiment

The polytunnel growth and fitness cost experiment analysed six seed families selected from the Renville population: 141, 29, 153, 1, 90 and 315. *A. tuberculatus* plants were grown in the absence of glyphosate application. Three cohorts of plant were grown to allow life history trait assessment over time. Harvest time points were undertaken, 47 days after transplanting (DAT) harvest 1 (H1), 75 DAT harvest 2 (H2) and 110 DAT harvest 3 (H3). H1 was conducted during the early growth phase, H2 was conducted when 50 % of plants displayed a floral meristem and H3 was conducted when plants displayed symptoms of senescence. The life history trait measures that were taken at each harvest time point are listed in table 5.1.

Plants were produced as outlined in section 3.2.1.1, seven days after germination seedlings (3-4 cm high) were transplanted into 1 L pots for H1 or 4 L pots for H2 and H3. Pots were filled with medium grade sphagnum moss peat 100 % (pH to 5.5 – 6.0; N = 800, P = 600, K = 800 µg g^{-1}). The experiment was conducted between June and September in a polytunnel with an average temperature of 18 ^{o}C (figure 5.3). H1 plants were arranged in a randomised block design separate to H2 and H3. H2 and H3 plants were laid out in a single randomised block design. The H1 layout contained 32 blocks such that 32 replicate plants were assessed per seed family to produce a total of 192 plants. The H2 and H3 layout contained 33 blocks so that 33 replicate plants were assessed per seed family per harvest to produce a total of 398

plants. H3 plants were re-randomised post H2 to maintain equal spacing between plants, however, the plant blocking was maintained. Life history traits were measured on plants as outlined in table 5.1.

Figure 5.3: Photo of harvest 2 & 3 growth and fitness cost plants from polytunnel experiment.

Table 5.1: Life history trait measures and calculated values for each harvest time point in glasshouse and polytunnel growth and fitness experiments. Harvest index (HI) = reproductive biomass/ total dry biomass) Stem to weight ratio (SWR) = stem dry biomass / total dry biomass Leaf to weight ratio (LWR) = leaf dry biomass /total dry biomass: Relative growth rate (RGR) (Eq. 5.1 & 5.2) *indicates measures which were only taken on plants where floral meristems had developed.

Harvest 1	*Harvest 2*	*Harvest 3*
Height	Height	Height
Stem diameter	Stem diameter	Stem diameter
Leaf number	Leaf number	Sex of individuals
Fresh total above ground biomass	Sex of individuals*	Fresh total above ground biomass
Dry total above ground biomass	Fresh total above ground biomass	Dry total above ground biomass
Leaf biomass	Leaf biomass	Leaf biomass
Stem biomass	Stem biomass	Stem biomass
	Reproductive biomass*	Reproductive biomass

$$\hat{r}_2 = \frac{\overline{\ln(W_2)} - \overline{\ln(W_1)}}{t_2 - t_1}$$

(5.1)

Relative growth rate: W denoted the biomass in grams at time point 1 and 2, t denotes the time in days at time point 1 and 2 (Hoffmann & Poorter, 2002)

$$V(RGR) = \frac{V(\overline{\ln W_2}) - V(\overline{\ln W_1})}{(t_2 - t_1)^2}$$

(5.2)

The variance for relative growth rate (Causton & Venus, 1981; Vila-aiub *et al.*, 2005).

5.2.3.3 Glasshouse growth and fitness experiment

Growth and fitness cost experiment two analysed eight seed families selected from the Renville population: 141, 29, 21, 153, 1, 90, 226 and 315. *A. tuberculatus* plants were grown in the absence of glyphosate application. Harvest time points were undertaken, 27 d (H1), 47 d (H2) and 87 d after transplanting (H3). Harvest times preceded those of polytunnel growth and fitness experiment due to the comparatively high relative growth rates under glasshouse conditions (section 5.3.5). H1 and H2 were conducted at plant life history time points equivalent to those stated in the polytunnel growth and fitness cost experiment, however, H3 was taken at a later life history time point, after seed maturation. The life history trait measures, taken at each of the harvest time points, are listed in table 5.1.

Plant production was conducted as outlined in section 3.2.2.1. Seedlings were transplanted seven days after germination (3-4 cm tall) into 9 cm square pots containing a ratio 2:1 of top soil: medium grade sphagnum moss peat 100 % (pH =7.6, K= 176.3, P= 80.4, NO_3= 146.6, mg = 377.9 µg g^{-1}). Plants were grown in a glasshouse heated to 23/ 18 ^{o}C (16:8 hr; day/ night, with ventilation at +2 ^{o}C above set points), with supplementary lighting (figure 5.4). H2 and H3 plants were transferred into 4 L pots 28 days after transplanting. Plants from all harvests were contained within ten randomised blocks arranged down the length of the glasshouse. The blocks mitigated any environmental temperature gradient produced by the position of the glasshouse door. Plants were re-randomised post H1 and H2 to maintain equal spacing between plants, however, plants were kept in the same blocks. 20 replicate plants were produced per seed family per harvest with two replicate plants per block. Life history traits were measured on plants as outlined in table 5.1.

Figure 5.4: Photo of H3 glasshouse growth and fitness cost experimental plants.

5.2.4 Analysis

5.2.4.1 Dose response analysis

The dose response experiment conducted on the seed families selected for the polytunnel growth and fitness cost experiment (outlined in 5.2.1) was analysed as outlined in section 2.2.4 and 3.2.8 (5.3.1). Subsequently, a correlation analysis was performed on resistant indices (Eq. 2.1) from the two replicate dose response experiments (3.3.3 & 5.3.1, figure 5.5) to determine whether the initial dose response experiment provides a valid measure of resistance (5.3.1).

Student t-tests were performed on seed family LD_{50} values from section 3.3.3 & 5.3.1 to determine if seed families represent distinct resistance level (5.3.1).

5.2.4.2 Gene copy number mixed model analysis

Gene copy number was elucidated for 43 experimental and 6 control plants, using the cycle threshold (CT) values produce in the Q-PCR analysis. CT values were calculated for the gene of interest (*EPSPS*) and also the house keeping gene (*ALS*) in each technical replicate well (three/ four) for each plant. A mixed model analysis was performed to moderate the impact of variation observed between technical replicate CT well values. This mixed model was fitted using the restricted maximum likelihood (REML) algorithm in Genstat.

The mixed model used delta cycle threshold (Δ CT) values, these values indicate the difference between the house keeping gene mean CT and the gene of interest mean CT. The Δ CT values were produced through two steps: 1.The geometric means of

individual plant CT values were calculated for each plant across the three/four technical replicate wells for the gene of interest and the house keeping gene. 2. Δ CT values were calculated using the CT geometric means for the gene of interest and the house keeping gene for each plant as outlined in equation 5.3.

$$\Delta CT = \frac{\bar{x}_{EPSPS\ CT}}{\bar{x}_{ALS\ CT}} \tag{5.3}$$

The delta cycle threshold (Δ CT) is the geometric mean of the *EPSPS* cycle threshold (x̄ EPSPS CT) values of a plant across the three/four technical-replicate wells on a single plate divided by is the geometric mean of the equivalent *ALS* cycle threshold values (x̄ ALS CT).

Multiple replicate Q-PCRs optical plates were conducted for each plant. Δ CT values were calculated for each plant on each plate to produce a total of 195 Δ CT values representing 43 experimental and 6 control plants. Each plant had multiple Δ CT values assigned to them as they were measured on numerous replicate plates.

The factors that were included in the model are outlined below:

VARIABLES

- Calculated Δ CT values (Eq. 5.3) (response variable)
- Calculated standard deviation Δ CT values (Eq. 5.4) (weighted variable)

FIXED EFFECTS

- Plant identification number (treatment factor)
- Seed family identification number (treatment factor)

RANDOM EFFECTS

- Optical plate each Δ CT estimate come from (blocking factor)
- Machine each optical plate was measured in (blocking factor)

The mixed model was used to calculate the mean Δ CT of individual plants and the mean Δ CT values of seed families using the 195 calculated Δ CT values (response variable). The fixed (treatment) factors of the mixed model were plant identification number and seed family identification number. To determine the Δ CT values of seed families the plant treatment factor was nested in the seed family treatment factor. The random (blocking) factor of the mixed model was the optical plate nested within the machine. The random terms of the model controlled for any variation between the replicate optical plates and the two machines used. The observed Δ CT values were weighted using the inverse of the variance calculated from the Δ CT standard deviation (weighted variable) for each optical plate, across three/four plant technical replicate wells for each primer set (Eq.5.4).

$$\sigma_{\Delta CT} = \sqrt{((\sigma_{EPSPS\ CT})^2 + (\sigma_{ALS\ CT})^2)} \tag{5.4}$$

The standard deviation of the delta cycle threshold (σ Δ CT) is the square root of the standard deviation of the *EPSPS* cycle threshold replicate values of a plant across the three/four technical-replicate wells on a single plate, squared, plus the standard deviation of the *ALS* cycle threshold for the equivalent *ALS* replicate values, squared.

Therefore, the plant Δ CT values that were associated with a low Δ CT standard deviation would have a high influence on the final plant average Δ CT. Finally, the

gene copy number (GCN) was calculated using the mean Δ CT values that were produced for the mixed model analysis through taking the Δ CT as negative exponent to the base two ($2^{-\Delta CT}$), subsequently, each relative difference was expressed as a proportion of lowest Δ CT, which was assumed to have a gene copy number of one.

Wald statistics were produced to determine if there was a significant difference between the average gene copy number of each seed family. Additionally, Wald statistic's determined whether the variation between seed families and between plants within seed families is large relative to the background variation, between observations. Gene copy data was analysed in GenStat (13^{th} Edition).

5.2.4.2 Gene copy number and resistance

A regression analysis was performed to determine the relationship between average seed family gene copy number and LD_{50} resistance. The regression analysis was performed in R (R version 2.15.1: 2012-06-22) (R Development Core Team, 2009) using the nlme package version 3.1-109.

5.2.4.3 Gene copy number and Life history trait

Regression analyses were conducted on average seed family mean gene copy number and individual life history trait values as outlined in the preceding section. Figures show the mean gene copy number and mean life history trait relationships. A value was calculated to indicate the magnitude of the relationship between gene copy number and biomass was calculated using the parameters from the line equation produced in the regression analysis:

$$y = mx + b$$

(5.5)

The linear equation provided by a regression analysis: y is the growth or fitness measure, x is the gene copy number, b is the y axis intercept and m is the slope.

The gene copy number (GCN) required to produce a 10% reduction in plant growth calculated using parameters from the the regression analysis equation (above) where m is the slope and b is the intercept at the y axis.

$$GCN\ for\ 10\ \%\ fitness\ reduction = 0.1\left(\frac{b}{m}\right)$$

(5.6)

Furthermore, an analysis of individual gene copy number and life history traits was conducted on the 43 genotyped individuals of the 198 plants from H3. Tests were conducted with and without the single extreme individual containing a very high gene copy number. This dual analysis accounted for any leverage effect.

5.2.4.4 Life history trait and resistance

A Pearson's correlation analysis was performed on normally distributed data. A correlation analysis was performed, in preference to a regression analysis because the independent variable needs to be causative in order to conduct a regression analysis. Resistance may be associated with fitness; however, resistance does not cause a reduction in fitness. A log transformation was performed on data provided

the transformation normalised, the distribution of the data. Spearman's rank correlation was performed on non-normal distributed data which could not be transformed. The mean LD_{50} seed family estimates were correlated against individual life history trait data sets (Polytunnel: 196 df Glasshouse: 158 d.f.). Figures show the mean seed family LD_{50} and mean life history trait relationships. All correlation analysis was performed in R (R version 2.15.1: 2012-06-22) (R Development Core Team, 2009) using the Hmisc package version 3.1-109.

5.3 Results

5.3.1 Comparison of seed family dose response one and two

The resistance levels of the seed families observed in the two dose response experiments are depicted in figure 5.5. This experiment supplemented and confirmed the LD_{50} values produced in the initial dose response experiment (section 3.3.3). The six Renville seed families selected for the polytunnel growth and fitness cost experiments (1, 90, 315, 153, 141 and 29) and the two addition seed families selected for the glasshouse growth and fitness cost experiment (21 and 226) have been selected from the clone protocol selected from the dose response conducted in section 3.3.3. The LD_{50} measures obtained from the seed family dose response one (3.3.3) were used as the resistance measure for the fitness cost analyses.

The resistant indices obtained in the initial seed family dose response were correlated against the corresponding resistance indices obtained from the repeat seed family dose response (figure 3.17). There was a significant positive relationship ($P<0.01$, $r_4=0.925$) between resistant indices for the two dose response experiments (figure

5.6). Moreover, the seed family resistant index ranks for the two dose response experiments correspond, with the exception of two seed families. The resistant indices for seed family 153 and 141 have switched rank however; the overlapping standard error suggests the two seed families were not distinct from one another and therefore resistant index ranks were interchangeable. The two dose response experiments produced consistent results and therefore, the LD_{50} values from the initial seed family dose response can be used to represent the seed family resistance levels.

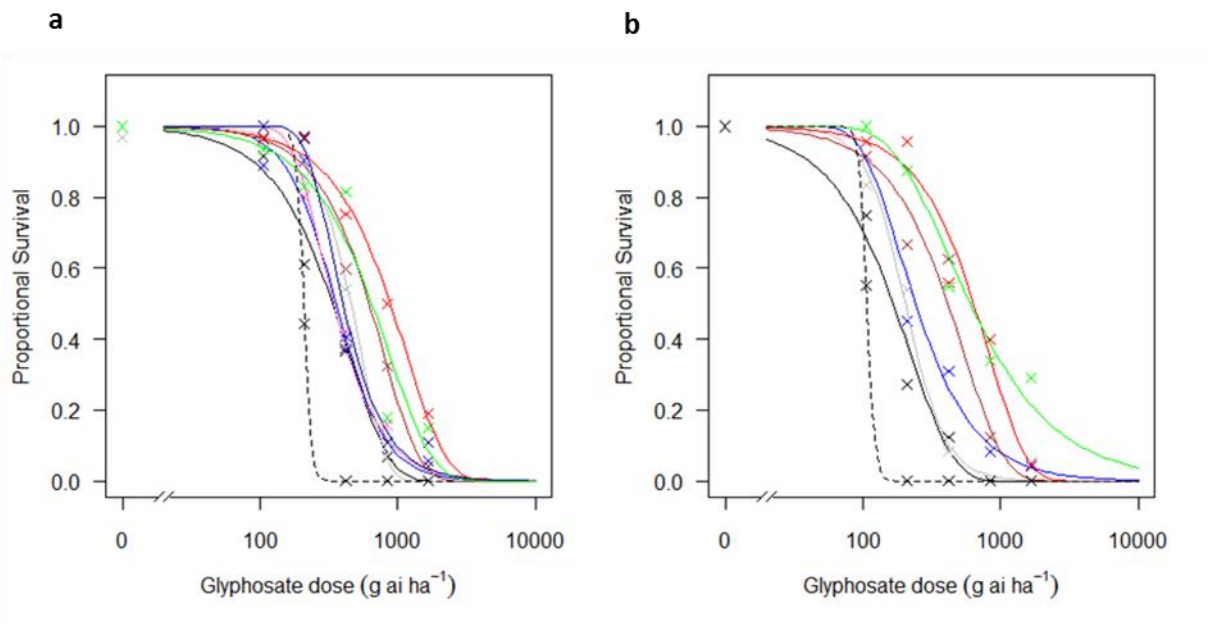


Figure 5.5: Renville seed family dose response for growth and fitness cost experimental seed families. a. Proportional survival of eight *A. tuberculatus* Renville seed families from dose response one (DR 1) after glyphosate application. **b. Proportional survival of six *A. tuberculatus* Renville seed families from dose response two (DR 2) after glyphosate application.** The colours correspond to seed family: 1 (x), 315 (x), 153 (x), 90 (x), 141(x), 21 (x), 226 (x) and 29 (x). Solid lines indicate the seed families and the dashed line indicates the standard sensitive population. Each of the models associated with the seed families has been fitted independently for DR1 (table 3.3) and DR2 (not shown).

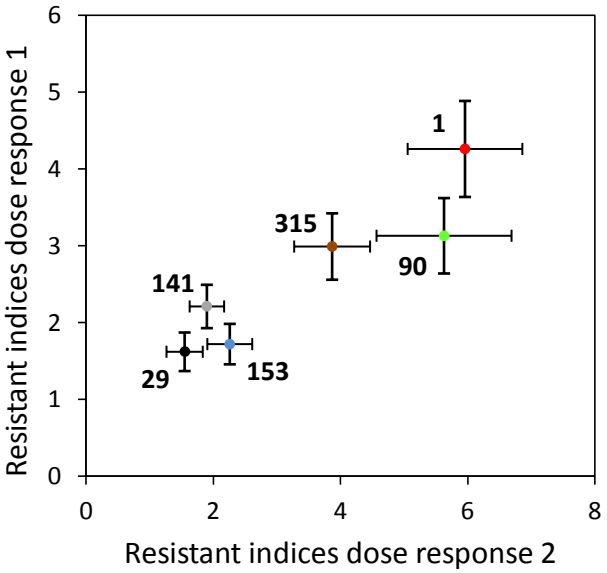


Figure 5.6: Association between seed family resistant indices (Based on the LD_{50} calculated using equation 2.2) for dose response 1 and 2. Numbers correspond to seed family numbers. Error bars are standard errors of the mean.

It is important that the seed family LD_{50} values used in the growth and fitness cost experiments provide discrete units for comparison. T-tests were performed on LD_{50} values for the selected polytunnel growth and fitness cost seed families. Ten out of the 15 LD_{50} comparisons indicated that seed families contained discrete resistance levels. The seed families used in the polytunnel growth and fitness cost experiment (table 5.2) showed significantly discrete LD_{50} values for all but the immediately adjacent seed families. Furthermore, comparison of the LD_{50} values for the second dose response experiment, exhibited significantly discrete LD_{50} values in 12 out of 15 comparisons (not shown). Therefore, the six seed families selected for the polytunnel growth and fitness cost experiment represent discrete points along a continuum of resistance.

Table 5.2: P-values for LD_{50} value pair comparisons (Student t- test) from seed family (SF) and S2 standard sensitive. Only seed families used in the polytunnel experiment are presented. LD_{50} values are taken from the initial dose response experiment conducted on seed families segregated using the clone protocol (3.3.3). Bold text indicates a non-significant difference between seed families LD_{50} values. A uniform log- logistic model (analysis not shown; Goodness of fit test, P= 0) was applied to the data to allow a comparison of seed family LD_{50} parameters. Seed families are aligned from left to right in LD_{50} value order.

	Resistant →					Susceptible
SF	1	90	315	141	153	29
1						
90	**0.073**					
315	*0.030*	**0.716**				
141	*0.000*	*0.034*	**0.053**			
153	*0.000*	*0.006*	*0.008*	**0.275**		
29	*0.000*	*0.000*	*0.000*	*0.019*	**0.306**	
SF	*0.000*	*0.000*	*0.000*	*0.000*	*0.001*	*0.011*

The two additional seed families were added for the glasshouse growth and fitness cost experiment: 21 and 226. The LD_{50}'s of seed family 21 and 226 were not associated with discrete LD_{50} values as no significant difference was observed between four and five adjoining seed family LD_{50} values, respectively (not shown). Although seed family 21 and 226 do not contain a discreet level of resistance, the

addition of these seed families in the glasshouse growth and fitness cost experiment can provide further insight into the impact of resistance on fitness.

5.3.2 Gene copy number & LD_{50}

The average seed family Δ CT values and relative *EPSPS* gene copy number are presented in table 5.3. There was a significant difference between the average gene copy number of each seed family (Wald statistic= 309.97, df=6, Wald df=51.66, X^2 P <0.001). Furthermore, the variation between plants within seed families was low relative to the background variation, between Δ CT observations (Wald statistic= 343.80, df=43, Wald df=8, X^2 P<0.001). There was a significant positive relationship between average seed family *EPSPS* relative gene copy number and seed family LD_{50} (figure 5.7) (f-statistic= 23.59; df=1; 4, P=0.0083, R^2 = 0.8188). The linear relationship suggests that one gene copy will increase resistance by an estimated 36 g ai ha^{-1}. In conclusion, amplified *EPSPS* gene copy number was associated with increased glyphosate resistance in the *A. tuberculatus* Renville population.

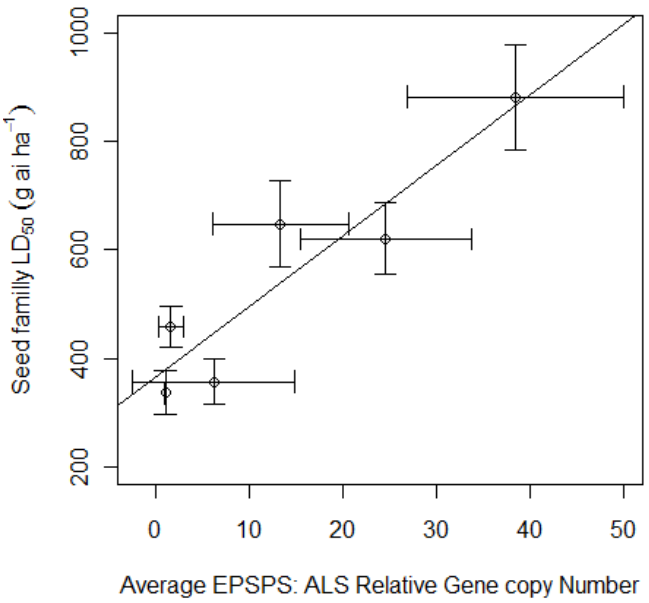


Figure 5.7: The relationship between seed family average *EPSPS* relative gene copy number and seed family LD_{50} (g ai ha^{-1}). Equation for the linear model: y = 365.47+13.03x. R^2 = 0.855, $F_{1,5}$= 23.59, P<0.01. Points represent mean values and error bars are standard errors of the mean encompassing the variation between plates and replicate plants.

Table 5.3: The average delta cycle threshold (Δ CT) values calculated for each seed family and the resulting gene copy number (GCN) relative to seed family 29 (with the lowest Δ CT). Numbers in brackets are standard errors of the mean.

Seed Family	*Average Δ CT value*	*GCN relative to seed family 29*
1	-3.37 (1.007)	38.85(11.610)
29	1.91 (0.1006)	1.00(0.053)
90	-1.836 (0.992)	13.42(7.249)
141	1.238 (0.995)	1.59(1.281)
153	-0.738 (1.021)	6.27(8.672)
315	-2.724 (1.002)	24.83(9.133)

5.3.3 Growth and fitness cost experiment Polytunnel

5.3.3.1 Biomass

5.3.3.1.1 Harvest 1

H1 individual dry biomass data was log transformed to normalise data distribution. H1 plants harvested 47 days after transplanting exhibited a weak negative trade-off between LD_{50} and biomass (r_{196}=-0.17, P<0.05) and gene copy number and biomass ($F_{1,190}$=6.66, R^2=0.035, P< 0.01, y=-0.0003x+0.062) (figure 5.8). The Pearson's correlation coefficient was low (0.17), indicating a weak relationship between the variables. Quantification of the extent of the growth penalty was calculated using the parameters determined form the regression model Eq.5.6. Thus, at the early growth stage (47 days after transplanting), 21 extra gene copies would lead to a 10 % reduction in biomass.

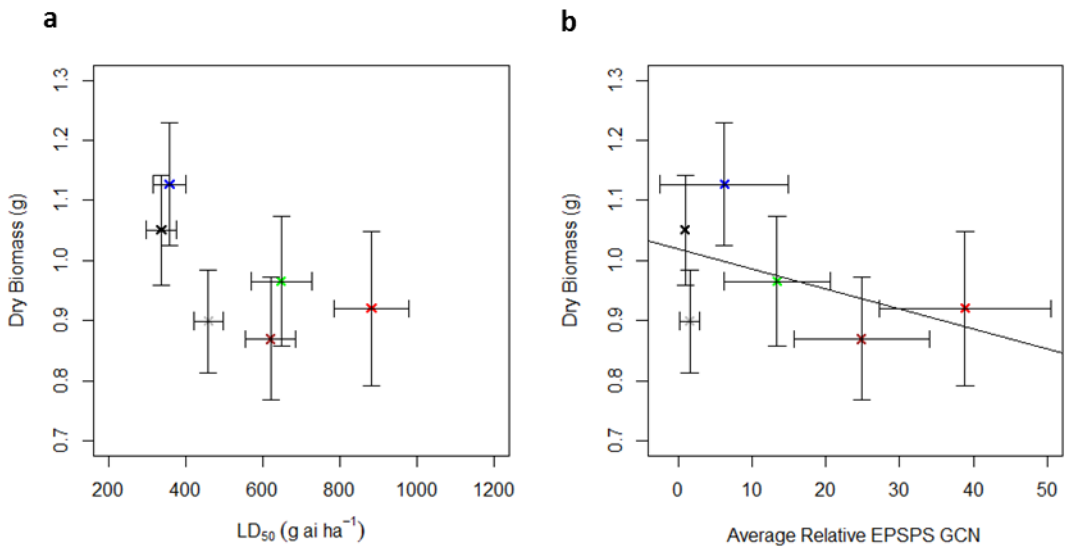


Figure 5.8: a. Dry Biomass and LD_{50} 47 days after transplanting Correlation statistics r_{196}=-0.17, P<0.05; **b. Dry Biomass and relative EPSPS gene copy number (GCN) 47 days after transplanting** regression statistics for $F_{1,190}$=6.66, R^2=0.035, P< 0.01. y=-0.0003x+0.062. Colours represent distinct seed families 1 (x), 315 (x), 153 (x), 90 (x), 141 (x) and 29 (x). Error bars are standard errors of the mean.

5.3.3.1.2 Harvest 2

Due to time constraints, dry biomass was not measured for H2. H2 plants were harvested 87 days after transplanting and exhibited no significant negative trade-off between LD_{50} and biomass (r_{196}=0.12, P=0.084) or gene copy number and biomass ($F_{1,190}$=2.125, R^2=0.011, P=0.146, y=-0.261x+137) (figure 5.9).

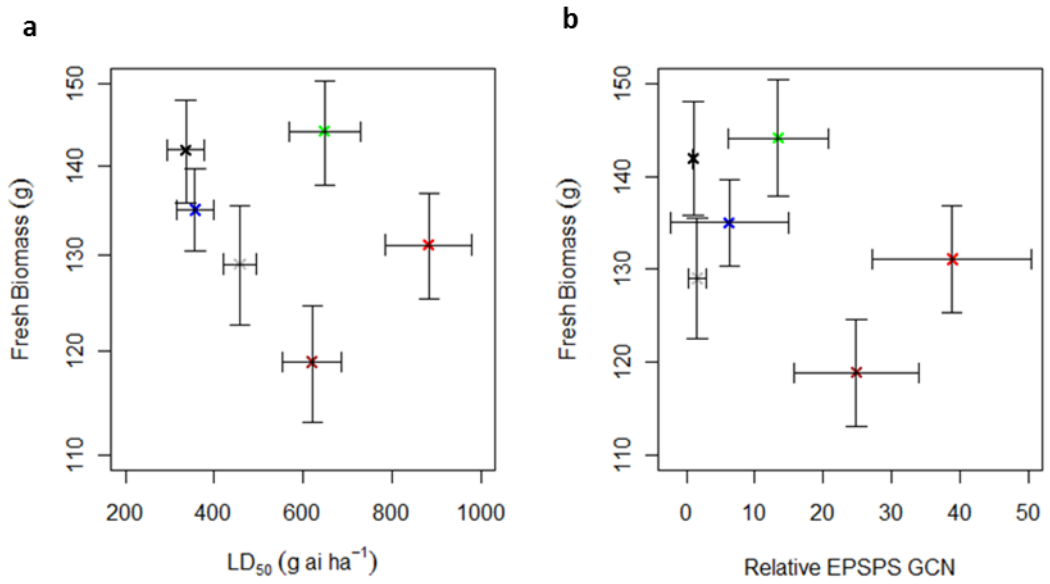


Figure 5.9: a Dry Biomass and LD_{50} 87 days after transplanting. Correlation statistics r_{196}=0.12, P=0.084; **b. Dry Biomass and relative EPSPS gene copy number (GCN) 87 days after transplanting.** Regression statistics: $F_{1,190}$=2.125, R^2=0.011, P=0.146, y=-0.261x+137. Colours represent distinct seed families 1 (x), 315 (x), 153 (x), 90 (x), 141 (x) and 29 (x). Error bars are standard errors of the mean.

5.3.3.1.3 Harvest 3

H3 individual biomass data was log transformed to normalise data distribution. H3 plants were harvested 110 days after transplanting exhibited a negative trade-off between LD_{50} and biomass (r_{196}=-0.19, P<0.05) and a negative trade-off between gene copy number and biomass ($F_{1,190}$=6.49, R^2=0.033, P<0.05, y=-0.0014x+1.604). The Pearson's correlation coefficient was low (0.19), indicating a weak relationship between the varriables. The reproductive stage (110 days after transplanting) indicated the 115 extra gene copies would lead to a 10 % reduction in fitness (figure 5.10).

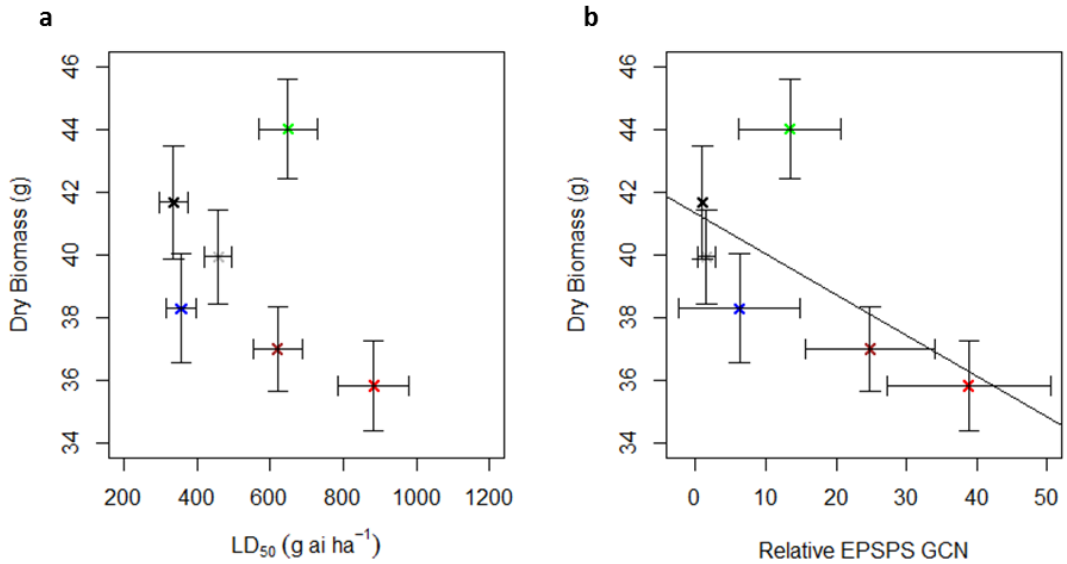


Figure 5.10: a Dry Biomass and LD_{50} 110 days after transplanting. Correlation statistics r_{198}=-0.19, P<0.01; **b. Dry Biomass and relative EPSPS gene copy number (GCN) 110 days after transplanting.** Regression statistics: $F_{1,190}$=2.20, R^2=0.011, P= 0.139. Colours represent distinct seed families 1 (x), 315 (x), 153 (x), 90 (x), 141 (x) and 29 (x). Error bars are standard errors of the mean.

5.3.3.1.4 Biomass Summary

The biomass data indicates that there was some evidence for a small growth penalty associated with resistance when plants were grown in the absence of competition; this relationship was particularly strong at the early growth stage (H1). Conversely no significant growth penalty was observed in H2 plants.

5.3.3.2 Fitness- Reproductive biomass

The fitness measure utilised for the polytunnel experiment was reproductive biomass 110 days after transplanting. H3 plants did not reveal a negative trade-off between LD_{50} and reproductive biomass production (r_{196} = -0.05, P= 0.52). Similarly, there

was no significant relationship between gene copy number and reproductive biomass (R^2=-0.028, $F_{1,4}$= 2.67, P= 0.18). This analysis indicates no fitness penalty was present when plants were grown in isolation (figure 5.11).

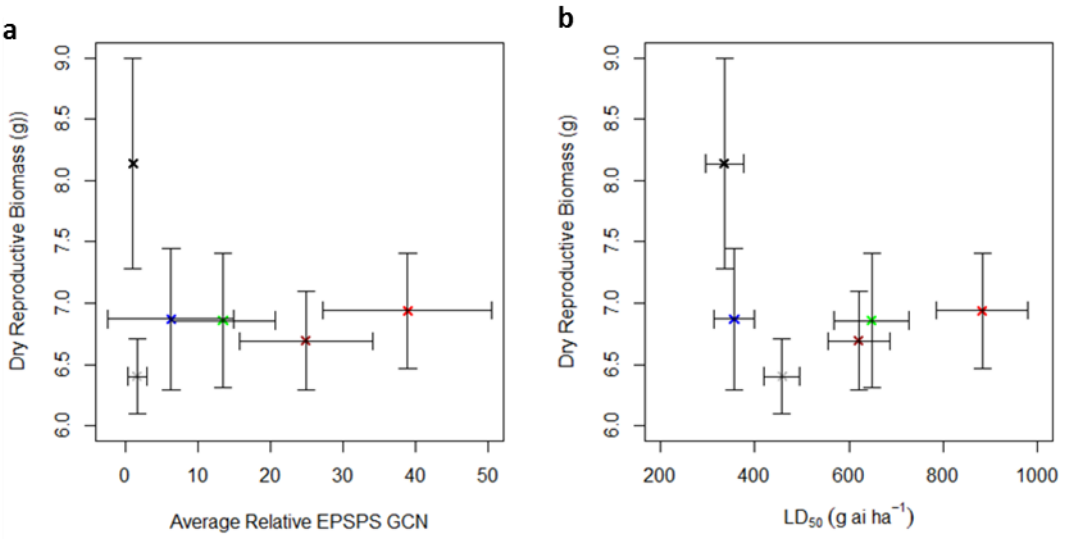


Figure 5.11: a. Reproductive biomass of seed families and LD_{50} 110 DAT. Correlation statistics (r_{198} = -0.05, P= 0.53) **b. Reproductive biomass of seed families and relative *EPSPS* gene copy number (GCN) 110 DAT**. Regression statistics R^2=-0.001, $F_{1,4}$=0.775, P= 0.38.Colours represent distinct seed families 1 (x), 315 (x), 153 (x), 90 (x), 141 (x) and 29 (x). Error bars are standard errors of the mean.

5.3.3.3 Individual plant gene copy number & Biomass

Relative *EPSPS* gene copy number was measured for 43 individual plants from the H3 cohort. This data allows the determination of the relationship between gene copy number and biomass on individual plants (figure 5.12).

There was a significant negative relationship between plant above ground dry biomass and *EPSPS* relative gene copy number 110 days after transplanting ($P<0.001$, $F_{1,41}= 36.61$, $R^2= 0.471$, $y=-0.043x+39.07$). The observed negative relationship was also significant when the extreme value was removed from the analysis ($P<0.001$, $F_{1,40}= 13.7$, $R^2= 0.255$, $y=-0.07x+40.08$). Furthermore, the slope of the relationship was steeper in the model without the extreme value (-0.07) compared to with the extreme value (-0.043). Specifically, a given number of gene copies would be predicted to reduce the biomass of a plant by a greater degree using the without extreme value model. The relationship between gene copy number and biomass indicates that an additional 91 gene copies would be required to reduce the biomass of a plant by 10% (57 without extreme value).

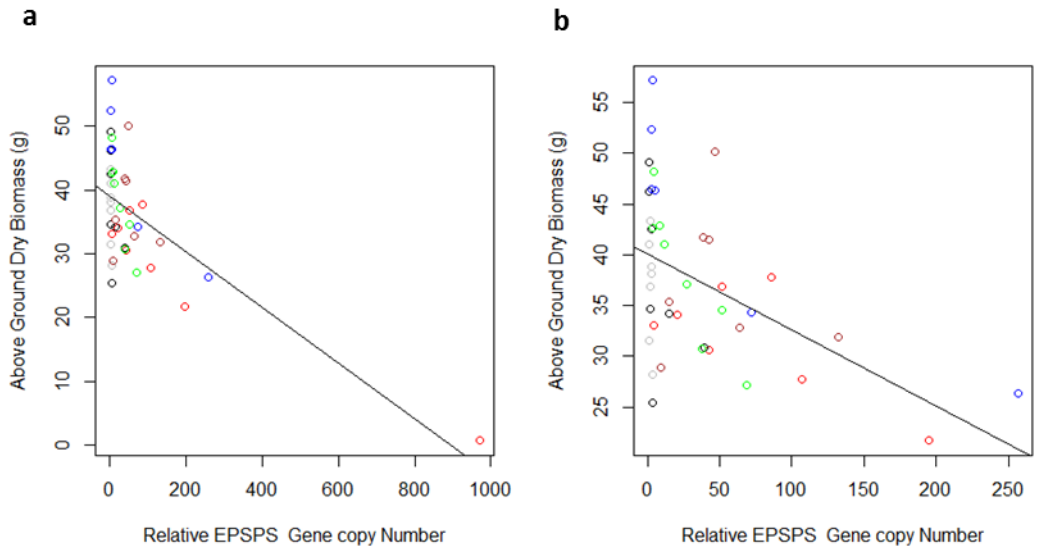


Figure 5.12: a. The relationship between total dry biomass and *EPSPS* relative gene copy number 110 days after transplanting with extreme value. Regression statistics with all data P<0.001, $F_{1,41}$= 36.61, R^2= 0.471, y=-0.043x+39.07, **b. The relationship between total dry biomass and *EPSPS* relative gene copy number 110 days after transplanting without extreme value.** P<0.001, $F_{1,40}$= 13.7, R^2= 0.255, y=-0.07x+40.08.Colours represent seed families: 1 (o), 29 (o), 90 (o), 141 (o), 153 (o) & 315 (o).

5.3.3.3.2 Relationship within seed family

The analysis outlined below determined whether the negative trade-off between gene copy number and biomass observed across seed families, was present within the individual seed families. A significant negative relationship between gene copy number and dry biomass was present within three out of six seed families (figure 5.13). However seed families 315 and 29 do not produce a significant negative relationship between gene copy number and biomass. Additionally, the distribution

of gene copy number values in seed family 141 does not represent a large spectrum of gene copy numbers found between seed families, therefore a negative trade-off was not anticipated.

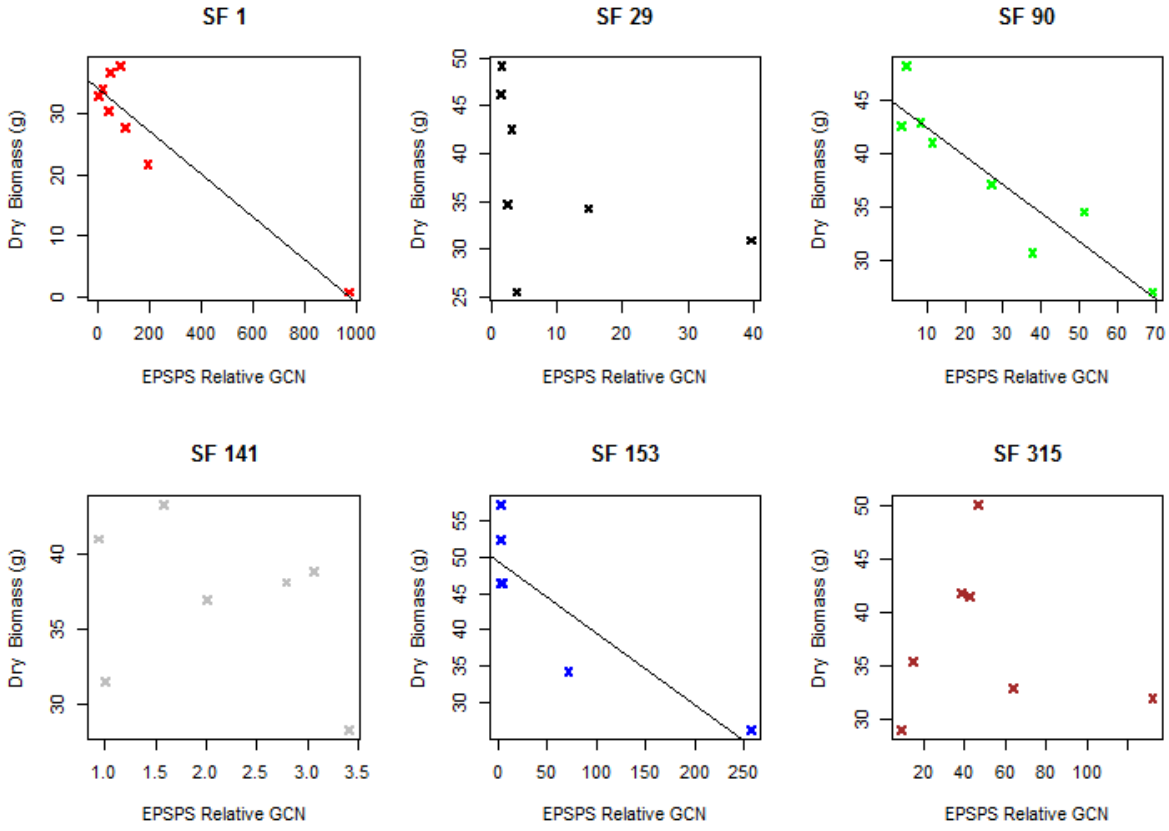


Figure 5.13: **Within seed family (SF) dry biomass and gene copy number (GCN) 110 days after transplanting.** Note the x axis scale varies depending on the gene copy number distribution. Colours represent seed families: 1 (x), 315 (x), 153 (x), 90 (x), 141 (x) and 29 (x). SF1 $F_{1,6}$=50.31, R^2=0.894, P<0.001, y=-0.035x+34.36; SF29 $F_{1,5}$=1.24, R^2=0.199, P=0.316; SF90 $F_{1,6}$=33.85, R^2=0.850, P<0.01, y=-0.266x+45.13; SF141 $F_{1,5}$=0.671, R^2=0.118, P=0.450; SF153 $F_{1,4}$=12.55, R^2=0.758, P<0.05, y=-0.099x+49.41; SF315 $F_{1,5}$=0.075, R^2=0.0148, P=0.795.

5.3.3.2 Stem diameter

Stem diameter can be used as a proximate measure to indicate plant size. Sequential measures were taken throughout the life span of H3 plants. The relationship between stem diameter and gene copy number at 47, 63, 90 and 110 days after transplanting is displayed in figure 5.14. Significant negative relationships between *EPSPS* gene copy number and stem diameter were observed at all time points. The early growth harvest data (H1) supports the sequential data: a significant relationship was observed between harvest 1 stem diameter and gene copy number ($P<0.001$, $F_{1,184}=11.88$, $R^2 = 0.061$, $y= -0.03x+6.66$) (not shown). However, the harvest two data did not support the sequential data: no negative relationship between stem diameter and gene copy number was observed at a late growth phase (H2) ($P=0.665$, $F_{1,195}=0.187$, $R^2 = 0.0009$, $y= -0.0059x+15.96$) (not shown).

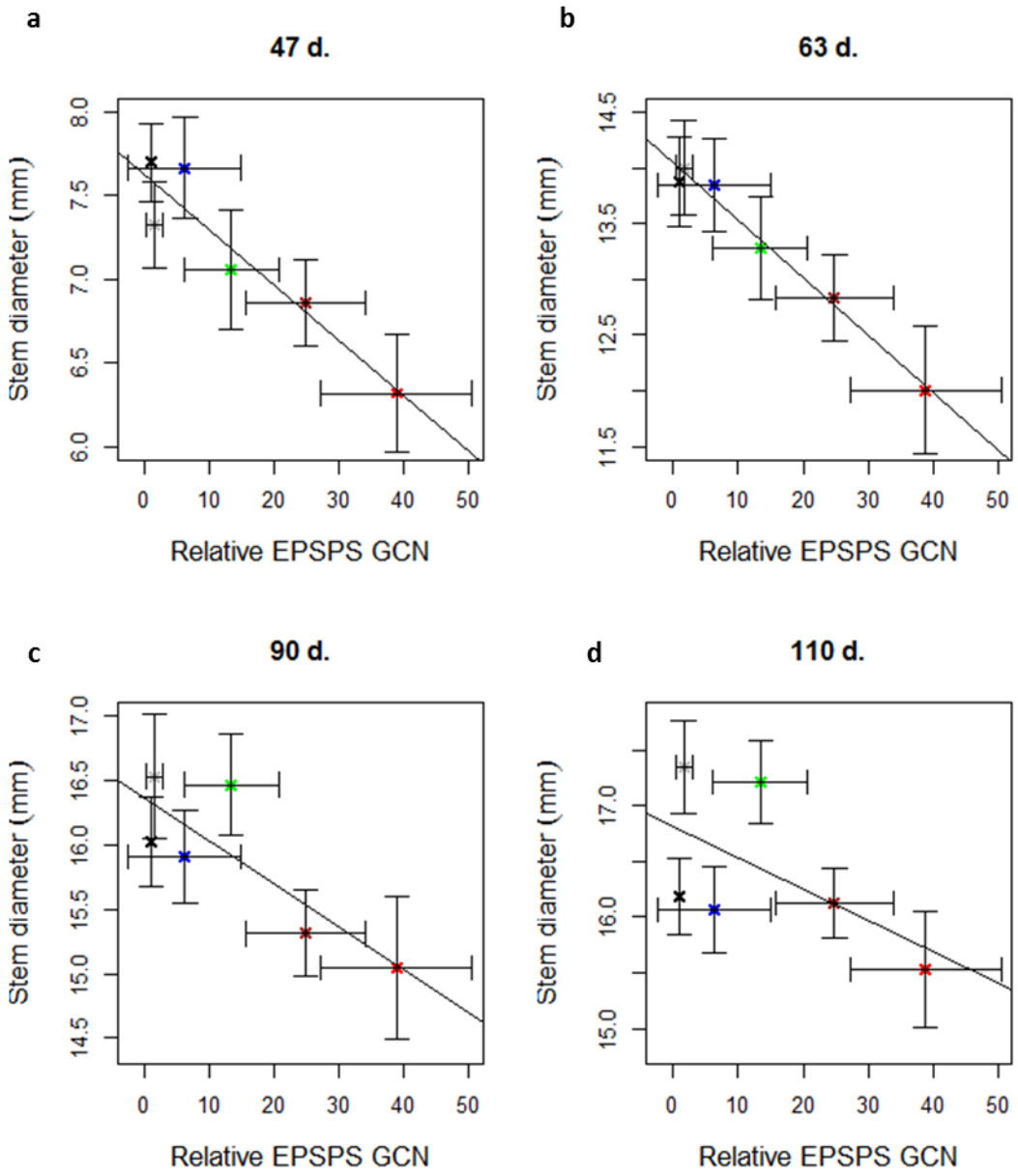


Figure 5.14: The relationship between the average relative *EPSPS* relative gene copy number and stem diameter at four time points for polytunnel growth and fitness cost experiment H3 plants. Regression analysis equations, P and R^2 values are presented for the mean values. Points are seed families: 1 (x), 315 (x), 153 (x), 90 (x), 141 (x) and 29 (x). Error bars are standard errors of the mean. **a.** 47 days after transplanting (DAT) P<0.0001, $F_{1,194}$ =14.1, R^2 = 0.067, y= -0.03x+7.63; **b.** 63 DAT P<0.0001, $F_{1,194}$ =14.92, R^2 = 0.071, y= -0.051x+14.04; **c.** 90 DAT P<0.01, $F_{1,194}$ =7.16, R^2 = 0.035, y= -0.034x+16.36; **d.** 110 DAT P<0.05, $F_{1,194}$ =5.29, R^2 = 0.026, y= -0.023x+16.81.

5.3.4 Glasshouse growth and fitness cost experiment

5.3.4.1 Growth - Biomass

5.3.4.1.1 Harvest 1

H1 plants harvested 27 days after transplanting produced a significant positive association between LD_{50} and biomass ($P<0.05$; $r_{160}= 0.21$) (figure 5.15). The Pearson's correlation coefficient was low (0.21), indicating a weak relationship between the varriables.

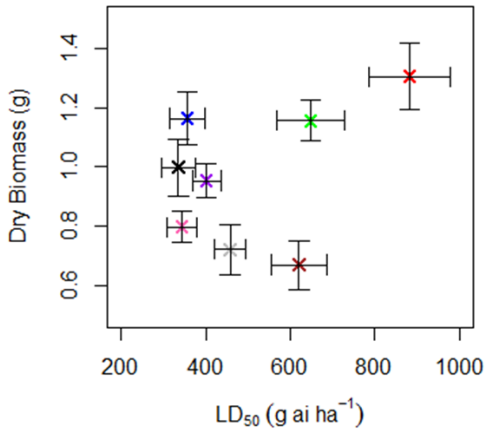


Figure 5.15: LD_{50} seed family dry biomass at 27 days after transplanting for the glasshouse growth and fitness experiment. Colours represent seed families: 1 (x), 315 (x), 153 (x), 90 (x), 141 (x), 21 (x), 226 (x) & 29 (x). Error bars are standard errors of the mean. Correlation statistics are $P<0.05$; $r_{160}= 0.21$.

5.3.4.1.2 Harvest 2

H2 plants harvested 47 days after transplanting produced a significant positive association between LD_{50} and biomass ($P<0.05$; $r_{160}= 0.19$) (figure 5.16). The Pearson's correlation coefficient was low (0.19), indicating a weak relationship between the varriables.

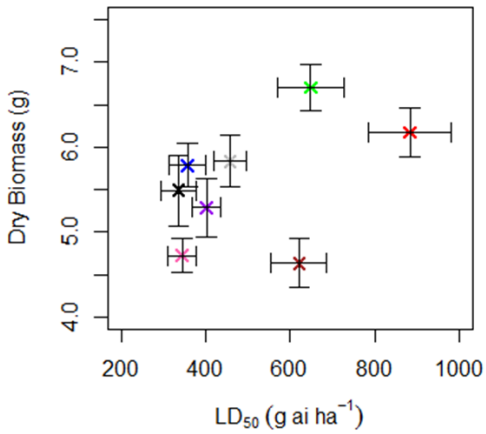


Figure 5.16: LD_{50} seed family dry biomass at 47 days after transplanting for the glasshouse growth and fitness experiment. Colours represent seed families: 1 (x), 315 (x), 153 (x), 90 (x), 141 (x), 21 (x), 226 (x) & 29 (x). Error bars are standard errors of the mean. Correlation statistics are $P<0.05$; $r_{160}= 0.19$

5.3.4.1.3 Harvest 3

H3 plants harvested 87 days after transplanting produced non-significant association, between LD_{50} and biomass (P=0.765; r_{160}= 0.02) (figure 5.17).

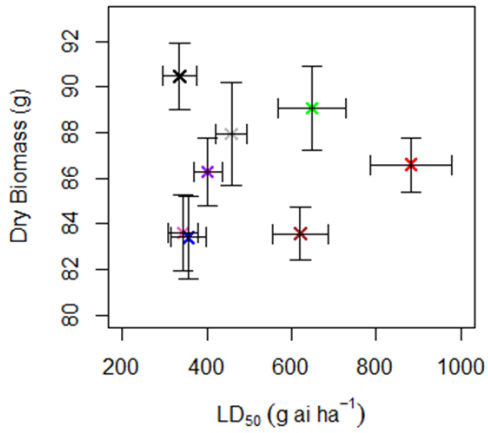


Figure 5.17: The relationship between seed family LD_{50} and dry biomass at 87 days after transplanting for the glasshouse growth and fitness experiment. Colours represent seed families: 1 (x), 315 (x), 153 (x), 90 (x), 141 (x), 21 (x), 226 (x) & 29 (x). Error bars are standard errors of the mean. Correlation statistics are P=0.765; r_{160}= 0.02.

5.3.4.1.4 Biomass Summary

The significant positive correlation between biomass and LD_{50} at H1 and H2 and the absence of a trade-off at H3 indicates that there was no negative trade-off between growth and resistance in a glasshouse environment. In fact, the significant positive correlations between biomass and LD_{50} for H1 and H2 indicate the potential for a resistance benefit in the absence of competition under glasshouse growth conditions.

5.3.4.2 Fitness- Reproductive biomass H3

H3 reproductive biomass production and LD_{50} did not reveal a negative trade-off 87 days after transplanting (figure 5.18). This analysis indicates that there was no fitness cost associated with resistance when plants were grown in isolation under a glasshouse growth environment.

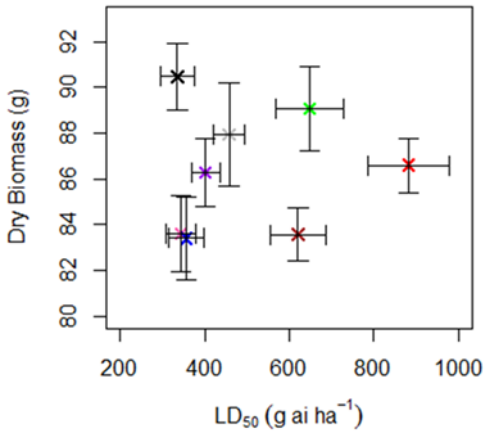


Figure 5.18: The seed family reproductive dry biomass at 87 days after transplantation. Colours represent seed families: 1 (x), 315 (x), 153 (x), 90 (x), 141 (x), 21 (x), 226 (x) & 29 (x). Error bars are standard errors of the mean. Correlation statistics r_6= 0.16, P= 0.71.

5.3.5 Relative Growth rate

There was no relationship between seed family relative growth rate and LD_{50} in the growth and fitness cost experiments conducted in the glasshouse and polytunnel (figure 5.19). Additionally there was no relationship between relative growth rate

and relative *EPSPS* gene copy number in the polytunnel growth and fitness cost experiment (not shown). The relative growth rate was significantly higher in the glasshouse experiment, ($P<0.001$, $F_{12}= 33.53$) this may be attributed to the supplementary lighting and heating within the glasshouse.

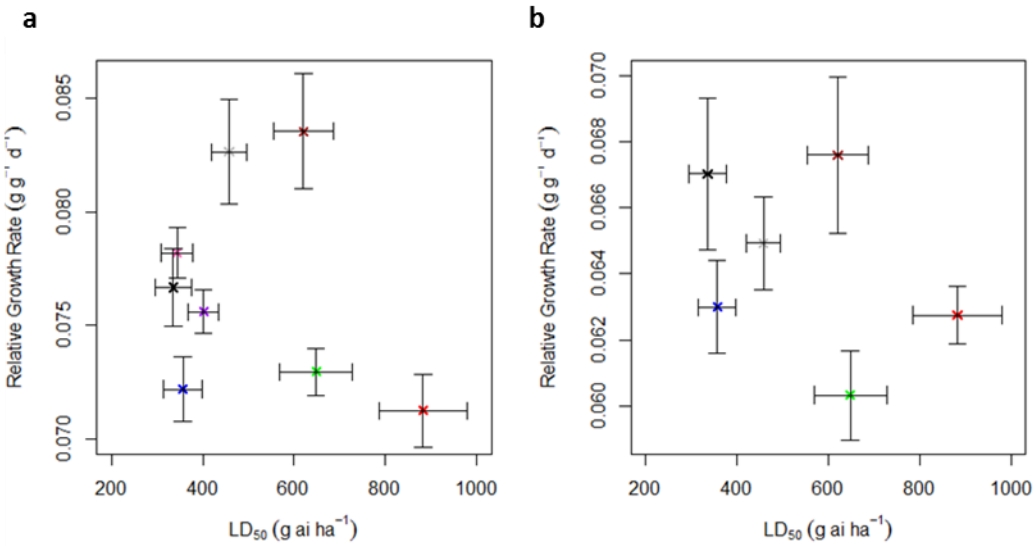


Figure 5.19: The association of seed family relative growth rate and LD_{50} for the (a) glasshouse (GH) between 27 and 87 days after transplanting and (b) polytunnel (PT) experiments between 42 and 110 days after transplanting. Colours represent seed families: 1 (x), 315 (x), 153 (x), 90 (x), 141 (x), 21 (x), 226 (x) & 29 (x). Error bars are standard errors of the mean. Correlation statistics for relative growth rate and LD_{50} are: GH 27-87 days after transplanting (DAT), r = -0.24, P= 0.56, df= 6 and PT 42-110 DAT r=-0.37, P= 0.47, df= 4.

5.3.6 Harvest Index

There was no association between seed family harvest index and LD_{50} in the glasshouse or the polytunnel growth and fitness experiments. Nor was there a relationship between harvest index and relative *EPSPS* gene copy number in the

polytunnel growth and fitness experiment (not shown). Therefore, there was no alteration in reproductive allocation associated with an increase in resistance when plants were grown in isolation under glasshouse and polytunnel growth environments (figure 5.20).

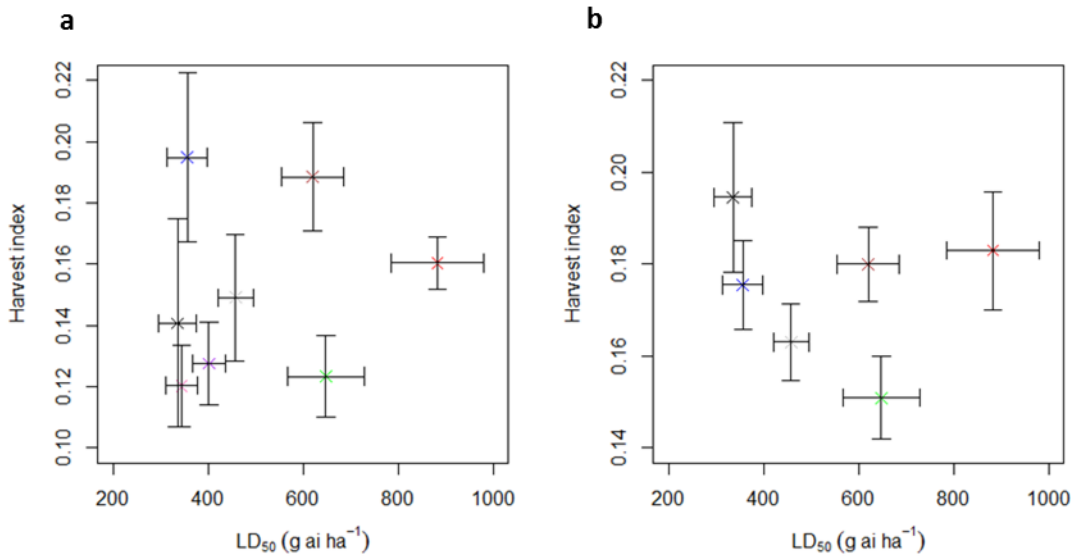


Figure 5.20: The association of seed family harvest index and LD_{50} for the (a) glasshouse (GH) 87 days after transplanting and (b) polytunnel (PT) experiment 110 days after transplanting, harvest 3. Colours represent seed families: 1 (x), 315 (x), 153 (x), 90 (x), 141 (x), 21 (x), 226 (x) & 29 (x). Error bars are standard errors of the mean. Correlation statistics for harvest index and LD_{50} are: 87 days after transplanting (DAT), r = 0.21, P= 0.61, df= 6, 110 DAT r= -0.26, P= 0.62, df= 4.

5.4. Discussion

5.4.1 Summary of results

5.4.1.1 Seed family resistance levels

There was a significant positive correlation between the seed family resistant indices produced in the glasshouse and polytunnel dose response experiments (5.3.1). This result indicates that the LD_{50} values were consistent when seed family resistance was quantified in a polytunnel and glasshouse environment. Furthermore, the seed family LD_{50} values indicated that each seed family utilised in the polytunnel growth and fitness experiment contained discrete resistance levels (when both dose response experiments are analysed). However, the additional seed families (21 and 226) utilised in the glasshouse experiment did not exhibit discrete LD_{50} values (5.3.1).

5.4.1.2 Seed family gene copy levels

The seed family gene copy numbers elucidated through Q-PCR and mixed model analysis were positively associated with seed family LD_{50} (5.3.2). Thus, *EPSPS* gene amplifacation was determined to be the primary mechanism of resistance within the Renville population. Furthermore, there was a significant difference between the average seed family gene copy number thus, seed families contain discrete gene copy numbers as units for comparison.

5.4.1.3 Seed family fitness

There was no association between seed family LD_{50} and reproductive biomass under polytunnel or glasshouse growth conditions. Similarly, there was no relationship

between *EPSPS* gene copy number and reproductive biomass under polytunnel growth conditions. These results indicate there was no fitness cost associated with resistance when plants were grown in isolation.

5.4.1.4 Seed family growth under polytunnel growth conditions

When seed families were measured under polytunnel growth conditions there was a significant negative relationship between total above ground dry biomass and LD_{50} (and gene copy number) at the early growth harvest (H1) and the reproductive harvest (H3) but no relationship was observed at a late growth harvest (H2). Similarly, negative relationships between stem diameter and gene copy number were observed at early growth harvest and the reproductive harvest but not the late-growth phase harvest. These results suggest there was a small growth penalty associated with resistance (and gene copy number) in a polytunnel growth environment. The relationship between gene copy number and biomass was weaker at the reproductive phase (H3) compared to the early growth phase (H1). However, the negative trade-off between individual plant gene copy number and biomass supports the negative relationship observed at 110 days after transplanting. The absence of a significant negative relationship at the late vegetative growth harvest (H2) may be due to a plant age effect on seed family growth.

Quantification of the extent of the growth penalty was achieved through calculating the percentage reduction in biomass associated with an amplified gene copy number. A 10% reduction in biomass was observed in plants with 21 extra gene copies at an early growth phase and a 10 % reduction in biomass was associated with 115 gene copies at the reproductive phase. These values indicate that the growth penalty was

ameliorated over time and that any extra gene copy numbers have a greater impact on biomass at an early growth stage. The average gene copy number across all plants tested was 35 gene copies (excluding the extreme individual) therefore, a decrease in biomass of 10 % associated with 21 extra gene copies may be observed in resistant individuals present in the field. No association was observed between relative growth rate (RGR) and LD_{50} or gene copy number; the lack of a RGR trade-off indicates that the trade-off in plant biomass was due to a low level of resistant plant vigour before the early growth harvest took place. A strong early growth penalty can be increased under conditions of competition to produce a fitness cost as plants with early vigorous growth dominate in a competitive environment (Paris *et al.*, 2006).

5.4.1.5 Seed family growth under glasshouse growth conditions

There was a significant positive relationship between total above ground biomass and LD_{50} at the early and late growth harvests under glasshouse growth conditions. These results suggest a growth benefit was associated with amplified *EPSPS* and potential explanations for this are outlined in section 5.4.4. Furthermore, these findings indicate that fitness associated with amplified gene copy number was influenced by abiotic conditions.

5.4.2 Evidence for fitness costs with the amplified gene copy numbers

It is important to consider the impact that a resistance mechanism may have on the fitness of a plant. Additionally, the potential fitness costs mechanisms that may arise in a resistant population can be determined by the resistance mechanism. The gene amplification resistance mechanism may potentially result in two fitness costs

mechanisms: resource divergence and sequence disruption. The constituent expression of the *EPSPS* gene copies may lead to an energetic drain on resources. For example, the constituent expression of salicylic acid in *Arabidopsis thaliana* provides resistance to *Peronospora parasitica* but produces a dramatically stunted phenotype (Mauch *et al.*, 2001; Heil & Baldwin, 2002).

The energetic costs associated with a doubling of transcription and translation rates were linked to a cost when studied in *Saccharomyces cerevisiae* (Wagner, 2005). Similarly, the constituent expression of the *E. coli* lactose operon, in the absence of lactose, is associated with a fitness cost (Koch, 1983; Stoebel *et al*., 2008). The mechanism of this cost was found to be due to the energetic costs associated with transcription (occupation of RNA polymerase and nucleotides) and/or translation (occupation of tRNA and ribsomes) (Stoebel *et al*., 2008).Conversely, the incorporation of amino acids into superfluous proteins and product accumulation had no affect on fitness. Therefore the cost of constituent expression was due to the procedure not the resulting product of the gene (Stoebel *et al*., 2008). These results indicate a potential selective pressure for gene regulation across the *E. coli* transcriptome (Stoebel *et al*., 2008) and other organisms. Additionally, the resource allocation theory may explain any costs associated with gene amplification or over expression resistant mechanisms. Indeed, Powles & Yu (2010) hypothesised that if all 100 plus *EPSPS* gene copies associated with glyphosate resistant *A. palmeri* individuals were constitutively expressed, then gene amplification should be associated with a fitness cost.

Secondly, the *EPSPS* gene may be inserted into essential DNA sequences which will disrupt the sequence function and result in an associated fitness cost (Mackay, 1989). The distribution of *EPSPS* gene copies throughout the *A. palmeri* genome provides an indication that sequence disruption may have occurred (Gaines *et al.*, 2010). For example, the gene amplification mediated insecticide resistance mechanism found in *Myzus persicae* (Peach-potato aphid) was associated with a maladaptive behavioural response which was mediated by a non-constituent fitness cost mechanism. *M. persicae* individuals containing the gene amplification of metabolic carboxylesterases genes exhibited decreased migration away from senescing leaves at low temperatures (Foster *et al.*, 2003). Notably, this negative trade-off was observed when the additional gene copies were not expressed, thus indicating the cost was not due to constituent expression (Foster *et al.*, 2003). These results highlight the potential for the mechanism of gene amplification to produce a non-constituent fitness cost.

Further to this, research on glyphosate resistant *A. palmeri* determined a positive relationship between *EPSPS* gene copy number and EPSPS protein level indicating constituent expression. Additionally, there was a positive relationship between shikimate accumulation and *EPSPS* gene copy number this indicated any gene copies in excess of 12, did not have a positive impact on the glyphosate resistance phenotype (Gaines *et al.*, 2010). Therefore, excessive EPSPS production beyond 12 gene copies was superfluous and in the presence of a fitness cost under persistent selection, the projected optimisation of 12 gene copies would occur.

5.4.3 Evidence against a fitness cost

The results from this study indicating a potential growth penalty associated with gene copy number in a polytunnel growth environment conflict somewhat with the fitness studies that have been conducted using *A. palmeri*. No relationship was found between *EPSPS* gene copy number and total above ground biomass in glyphosate resistant *A. palmeri* population form Georgia, USA (Goh *et al.*, 2013). Furthermore, the absence of a relationship between *EPSPS* gene copy number and above ground biomass was also found in a separate study investigating glyphosate resistant *A. palmeri* population (Ward & Giacomini, 2013). The results from the two above mentioned studies found no penalty associated with amplified *EPSPS* gene copy number in *A. palmeri* under optimum growth conditions.

The glasshouse and polytunnel growth and fitness experiments did not elucidate a fitness cost associated with resistance or gene copy number. These results support the absence of a relationship observed between gene copy number and seed production or harvest index (Ward & Giacomini, 2013).

Notably, the observed life history trait and gene copy number associations found in the study by Ward & Giacomini (2013) were strongly influenced by the genetic background of the seed families. Upon statistically controlling for the genetic background, no fitness cost or life history trade-off was associated with amplified *EPSPS* gene copy number (Ward & Giacomini, 2013). Therefore, glyphosate resistant *A. palmeri* containing a gene amplification mechanism of resistance will not reduce in frequency over multiple generations in the absence of glyphosate selection. Importantly these results show that the quantitative traits which segregate

independently from the resistance trait may influence seed family fitness to a greater extent than the fitness cost associated with the resistance allele and therefore the background genetic variation from the seed families may confound the elucidation of resistance trade-offs (Ward & Giacomini, 2013).

In this study, the relationships between gene copy number and plant biomass were analysed on a seed family basis, three out of six seed families exhibited negative relationships. Two seed families did not exhibit a relationship and one of the seed families did not exhibit gene copy values above four; therefore, no conclusion could be made for this seed family. These results suggest that the growth penalty was present in some seed families but not all. Further replication is required to conclude whether seed family genetic background has a greater influence on growth than the resistance trait.

5.4.4 Genotype and environmental interaction

The polytunnel experiment shows a negative trade-off between glyphosate resistance and dry biomass and stem diameter at early growth and reproductive harvests. The strength of this relationship was greater at early growth stages, however, no trade-off was found in the glasshouse growth environment.

In the glasshouse growth and fitness experiment, the most resistant seed families have average biomass levels equal to or greater than that of the susceptible seed families. However, in the polytunnel growth and fitness experiment, more resistant seed families have a lower or equal average biomass than more susceptible seed

families. This discrepancy in biomass across the two experiments has been attributed to a genotype-by-environment interaction.

The polytunnel had lower average temperature and no additional lighting; therefore it is possible that abiotic stress may lead to a reduction in photosynthate production and thus increase a growth penalty. The interaction between abiotic conditions may enhance or mitigate resistance costs (Koricheva, 2002; Vila-Aiub *et al.*, 2009a). Indeed, fitness costs experiments exhibited costs in controlled environments less frequently than uncontrolled environments (Koricheva, 2002) and the results of this study support this observation as the under controlled glasshouse growth conditions no resistance cost was observed, whereas under uncontrolled polytunnel growth conditions a resistance cost was observed.

A positive association was observed between biomass and LD_{50} for the early and late growth harvest in the glasshouse growth environment. These finding indicates a potential growth benefit under a high light and high temperature growth environment. A substantial fitness benefit was observed in *Oryza sativa f. spontanea* (Weedy rice) when a native *EPSPS* gene was over-expressed compared to susceptible individuals containing the same genetic background (Wang *et al.*, 2013). Indeed, 48–125% greater seed was obtained when plants were grown in intra and inter-phenotypic competition in the absence of glyphosate. Glyphosate resistant *Oryza sativa f. spontanea* individuals contained a higher concentration of tryptophan (a downstream product of the shikimate pathway), a higher level of photosynthesis, a greater seed number and higher germination. These fitness benefits were allocated to the cascading effect of *EPSPS* over production (Wang *et al.*, 2013). Such a cascading

effect may explain the weak positive relationship between resistance and biomass under high light conditions. Indeed, it is possible that a high light environment allows plants containing high EPSPS levels to utilise the excess downstream bi-products from the shikimate pathway. However, the same bi-products produced by the additional *EPSPS* gene copies would be superfluous under a low light environment due to limited energy resources. Therefore, it may be hypothesised that an energetic drain of extra gene copies occurs under low light and temperature levels; however, bi-product utilisation occurs under high light and temperature growth conditions. Indeed, previous studies have reported that the growth penalties associated with triazine resistance in weed species have been mitigated, amplified or reversed under different light and temperature conditions (Vila-Aiub *et al.*, 2009a). For example, Triazine susceptible *Brassica rapa* was a better competitor than the resistant phenotypes at low and medium temperatures and the susceptible phenotype was a better competitor at a high temperature (Plowman & Richards, 1997).

5.4.5 Conclusions

Average seed family *EPSPS* gene copy number was associated with seed family resistance, thus indicating that *EPSPS* gene amplification causes glyphosate resistance in the *A. tuberculatus* Renville population. No fitness cost was observed when plants were grown without competition, under glasshouse and polytunnel growth conditions. However, there was some evidence to suggest a growth penalty was present under polytunnel growth conditions. This is the first study to elucidate a growth penalty associated with *EPSPS* amplified gene copy number in an *Amaranthus* species. This growth penalty may be increased to produce a fitness cost

in a competitive environment (Van Dam & Baldwin, 2001; Vila-Aiub *et al.*, 2009a). This potential is investigated in chapter 6.

6.0 Assessing resistance trade-offs in *A. tuberculatus* plants in the presence of competition

6.1 Introduction

6.1.1 Plant competition

A plant has a zone of influence over which it can obtain resources; this zone is often modeled as a circle (Schwinning & Weiner, 1998; Weiner *et al.*, 2001). When two neighbouring plants have overlapping zones of influence, both plants will compete for the available resources in the intersecting region (Weiner *et al.*, 2001). Competition from a neighbouring plant can reduce the level of available resources and thus produce a reduction in plant growth or fitness, relative to a plants potential, in the absence of competition (Silvertown & Charlesworth, 2001; Park *et al.*, 2003). Larger plants are able to suppress the growth of neighbouring plants as they have a larger zone of influence and thus an advantage in obtaining the available resources (Weiner *et al.*, 2001).

There are two attributes associated with a plants competitive impact: the ability of a plant to respond to a depletion of resources caused by neighbouring plants (competitive response) and the ability of a plant to deplete the resources of neighbouring plants (competitive effect) (Goldberg, 1990; Vila-Aiub *et al.*, 2009b). If two species or phenotypes differ in their relative competitive effect and response, the better competitor will increase its relative frequency, and thus competitive interactions can impact the fitness of a species or phenotype (Weiner, 1990).

6.1.2 Size Asymmetric and symmetric competition

Plant competition may lead to size symmetric or asymmetric competition. In size symmetric competition a plant obtains resources in proportion to its size. Conversely, in asymmetric competition a plant obtains a greater proportion of resources relative to its size, thus enhancing the disparity between neighbouring plant size (Weiner, 1990). Consequently, plants with a vigorous early growth rate can suppress the growth of neighbouring plants by obtaining a greater proportion of resources and rapidly expanding their zone of influence. Therefore, if competition is size asymmetric one would expect to see an enhancement of any growth discrepancies observed in the absence of competition. Below ground competition is size symmetric because roots create depletion zones beyond which no additional nutrients can be obtained (Weiner, 1990). However, above-ground competition is asymmetric as the top leaves will intercept more light and light is a directional resource (Weiner, 1990). Asymmetric competition may be reduced by alterations in resource allocation and morphology or mediated by phenotypic plasticity. Tall plants have a competitive advantage through overtopping or placing leaves above shorter individuals. Indeed, the height to width ratio of a plant increases with density such that plants become taller and thinner with fewer lateral branches than plants grown in the absence of competition (Schwinning & Weiner, 1998). It has been found that high plasticity of stem diameter in response to competition can reduce the upper threshold before size asymmetric competition occurs (Schmitt *et al.*, 1987; Schwinning & Weiner, 1998; Weiner, 1990).

6.1.3 Plant phenotype competition

Variation in plant life history traits may impact a plant's fitness when grown under conditions of inter and intra-specific competition (Vila-Aiub *et al.*, 2009a). In particular, an early growth penalty associated with an herbicide resistance phenotype may be amplified in a competitive environment (Paris *et al.*, 2008). The early growth penalty associated with *A. tuberculatus EPSPS* gene copy number in the polytunnel growth and fitness experiment (section 5.3.3) may become greater and result in a fitness cost in a competitive environment.

6.1.4 Experimental approaches in plant competition studies.

There are a number of common methods utilised to analyse competition between plants: these include additive, response surface, replacement series and neighbourhood design experiments (Cousens, 1991; Park *et al.*, 2003). An additive experiment varies the density of one of the two competing species and as such, is highly relevant to an applied agricultural scenario where the optimum density of two species may be determined. For example, an additive design was utilised to maximise the land use efficiency through intercropping of *T. aestivum* and *Circer arietinum L*. (chickpea) (Banik *et al.*, 2006).

Alternatively, a replacement series design experiment varies the proportion of the two plant phenotypes or species at a constant density. Thus such experiments can determine which of the two plant types is better at competing for resources. For example, the relative competitive ability of an ALS susceptible *Amaranthus powellii* population and an ALS resistant population, containing the Trp-573-Leu target-site

mutation was determined using a replacement series experiment (genetic background variation not controlled) (Tardif *et al.*, 2006). However, replacement series experiments have been criticized for ignoring the potential for density dependent competition (Cousens, 1991).

Response surface design experiments allow a comprehensive analysis of the competitive ability of two plant types across multiple proportions and densities (Cousens, 1991). For example, the competitive ability of glyphosate resistant and susceptible *Lolium rigidum* phenotypes were compared using a response surface design experiment (Pedersen *et al.*, 2007). This design allows an estimate of intra and inter-phenotypic competition and the determination of how competition is effected by proportion and density.

Alternatively, a target-neighbourhood design experiment may be conducted to assess the effect of multiple different neighbouring species on a single target species at different densities or biomass (Weiner, 1982; Goldberg & Werner, 1983; Park *et al.*, 2003). A targeted-neighbourhood design was used to compare the relative competitive ability of P450 mediated herbicide resistant and susceptible *L. rigidum* phenotypes alongside *Triticum aestivum L.* (wheat) (Vila-Aiub *et al.*, 2009b).

6.1.5 Objectives

- Determine the relative level of inter and intra-phenotypic competition between R and S plants across a density and proportion competition response surface.

- Determine whether there is a negative trade-off between the level of glyphosate resistance (or *EPSPS* gene copy number) in *A. tuberculatus* seed families and growth or fitness under inter-specific competition.
- Determine whether there is a positive correlation between maize yield and *A. tuberculatus* seed family gene copy number and resistance level in a competitive environment.

6.2 Methods

6.2.1 Response surface experiment

The response surface experiment investigated the competitive ability of plants from relatively resistant and susceptible seed families obtained from the Renville phenotype segregations. Plant material was resistant seed family 9 (RI based on LD_{50} of 4.8) selected through glyphosate application to parental plants (section 3.2.6 and 3.3.4) and susceptible seed family 29 (RI based on LD_{50} of 1.7) selected in the clone phenotype identification (section 3.3.3). R and S plants were transplanted in a triangular grid (for example layouts see figure 6.1). Plants from R and S seed families were sown at a range of total densities and relative proportions as shown in table 6.1. Plants were produced as outlined section 3.2.2.1. Seedlings were transplanted into boxes 28.2 x 35.6 x 18.5 cm containing 13 kg of 2:1 top soil: Medium grade sphagnum moss peat 100 % (pH =7.6, K= 176.3, P= 80.4, NO_3= 146.6, mg = 377.9 µg/ g). Seedlings that did not transplant successfully were replaced up to 14 days after set up. Three replicates were established for treatments with total densities of 1100, 560, 300, 200 plants m^{-2} (figure 6.1 a-d), and six replicates were conducted for treatments containing a density of 100 plants m^{-2}

(figure 6.1 e) to increase the number of replicate plants for this treatment. Densities were selected to represent extreme low (100 plants m^{-2}) and high (1100 plants m^{-2}) density competition conditions. Boarder effect plants were arranged around the edges of the sample plant layouts at the same density and proportions as the experiment plants; these boarder effect plants were discarded and excluded from the analysis. A total of 66 boxes were produced across the 19 treatments. Boxes were arranged in three randomised blocks. Plants were harvested 114 days after transplanting. Measures of individual plant above ground fresh biomass, sex, height and stem diameter were recorded for experimental plants.

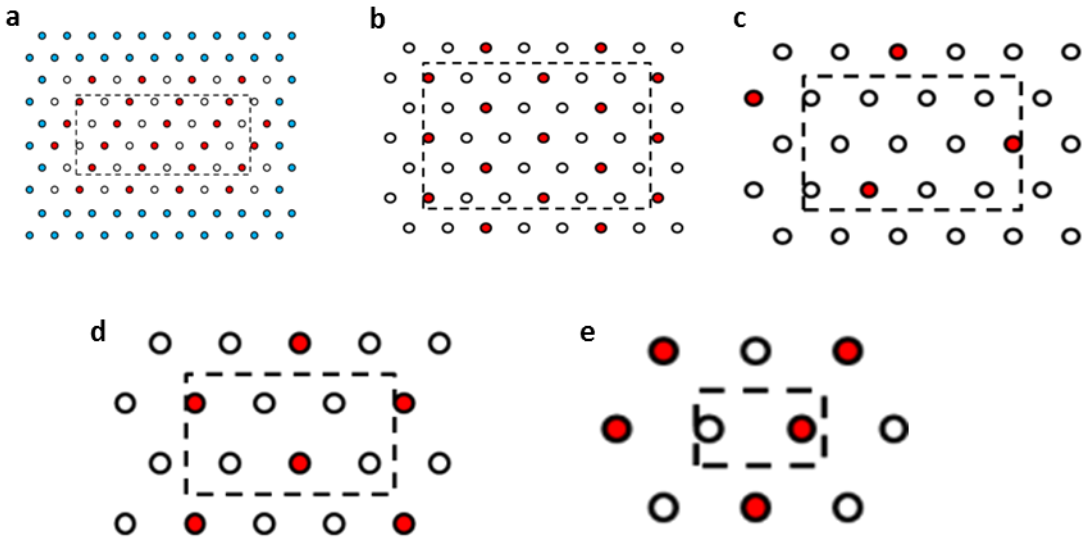


Figure 6.1: Examples of planting arrangement at selected densities and proportions of R and S seed families. a) R:S, 3:3 plant ratio, 1100 plants m^{-2}, **b)** R:S 4:2, 560 plants m^{-2} , **c)** R:S 6:1, 300 plants m^{-2}, **d)** R:S 4:2 200 plants m^{-2}, **e)** R:S 3:3 100 plants m^{-2}. Red and white spots represent the plant position for plants from the most resistant seed family (9) and the most susceptible seed family (29) phenotypes are interchangeable. Due to the high seedling volume required for the 1100 plants m^{-2} treatment, two rows of spacer plants (blue) were added to the outside of the layout, spacer plants were from seed family 153 (section 3.3.3). Plants within the dashed line box were harvested, plants outside of the box acted as edge effect plants.

Figure 6.2: Photos from the response surface design competition experiment a) Plants at density 100 plants m^{-2}, **b)** Example of layout of replicate boxes 48 days after transplanting **c)** Plants at 107 days after transplanting.

Table 6.1: The plant density and proportion selected for the response surface competition experiment. + indicates a treatment with a given density at a given resistant: susceptible (R: S) ratio which has been selected for analysis.

	Density (plants m^{-2})				
R:S	1100	560	300	200	100
6:0	+	+	+	+	+
6:1			+		
4:2		+		+	
3:3	+		+		+
2:4		+		+	
1:6			+		
0:6	+	+	+	+	+

6.2.1.1 Statistical Analysis

As mentioned above, plants that had died due to unsuccessful transplantation were replaced up to 14 days after transplanting; therefore, plants that died after this point were deemed to have died as a result of competition and thus included in the analysis as having zero biomass. Biomass data was analysed in R (R version 2.15.1: 2012-06-22) (R Development Core Team, 2009) using the nonlinear least squares (nls) function in the stats package version 3.0.1.

The raw biomass data had non-homogenous variance therefore the analysis was conducted on log transformed data. The model depicted in equation 6.1 was applied to R and S biomass data separately and the resulting *a*, *b* and *c* parameters were compared using z-tests. Furthermore, each *c* parameter was tested for a difference from one using a t-test. A *c* parameter of one indicates that the two phenotypes are equally competitive; therefore the t test determined whether phenotype i was significantly more or less competitive than the alternate phenotype j.

$$\log(y_i) = \log\left(\frac{A}{1 + B_i\left(N_i + C_{ij}\,N_j\right)}\right) + \varepsilon$$

(6.1)

Hyperbolic curve formula to describe the relationship between plant biomass, plant proportion and density. Y is the average plant above ground dry biomass. Where N is the density of phenotype *i* or *j*. ε is the error term. Parameter *a* denotes the maximum mean plant biomass in the absence of competition. Parameter *b* indicates the competitive response of a phenotype to an increase in density. Parameter *c* (also called the substitution rate) denotes the relative competitive effect of the phenotype. The *c* parameter value of 1 indicates that the two phenotypes have an equal competitive effect; > 1 indicates *i* is a poor competitor compared to *j* and < 1 indicates *i* a strong competitor compared to *j* (Pedersen *et al.*, 2007).

6.2.2 Neighborhood design experiment

The *Z. mays* (maize) and *A. tuberculatus* neighborhood design experiment investigated the potential for a trade-off between *A. tuberculatus* resistance and fitness under inter-specific competition.

The Renville *A. tuberculatus* seed families 1, 29, 90, 141,153, 315 (section 3.2.3) were chosen to represent a range of glyphosate resistance levels. The three pot layouts were: four *A. tuberculatus* plants from the same seed family around a central maize plant, four *A. tuberculatus* plants from the same seed family and maize grown in isolation (figure 6.4). The two *A. tuberculatus* pot layouts were conducted for the six seed families and a solitary maize treatment; there was a total of 13 treatments.

Ten replicate pots were produced for each treatment, with the exception of maize grown in isolation where 20 replicate pots were produced. Therefore, the 13 treatments produced a total of 140 pots. Plants were arranged in ten randomised blocks down the length of the polytunnel to mitigate any environmental gradient down the tunnel.

A common agricultural variety of maize (Kangaroo) was germinated in seed trays (15 x 20 cm) on 2.5 cm of sharp sand. Maize seed was placed between layers of absorbent paper and covered with 0.5 cm of sharp sand. Seed trays were watered, left to drain and placed in a polypropylene bag to minimise evaporation. Seed trays were kept in glasshouse growth conditions at 23/18 ^{o}C (16:8 hr; day/ night) with ventilation at +2 ^{o}C above these set points. *A. tuberculatus* seed was germinated 2 weeks before transplanting as outlined in section 3.2.1.1.

Maize and *A. tuberculatus* seedlings were transplanted as indicated figure 6.4. Maize was transplanted one week after sowing, into the centre of 4 L pots containing 2 kg of 2:1 top soil: Medium grade sphagnum moss peat 100 % (pH =7.6, K= 176.3, P= 80.4, NO_3= 146.6, mg = 377.9 µg/ g); weed seedlings were transplanted at the V1 corn growth stage, so that cotyledons were 2 cm above the soil surface. Unsuccessful transplanted weed seedlings were replaced up to 1 week after the initial transplant event so that all pots contained the desired plant number. *Amaranthus* seedling emergence timing equated to the VE maize stage due to transplant timings, this allowed an estimate of the maximum competitive effect of *A. tuberculatus* on maize. The experiment was conducted in a polytunnel from June to September under ambient conditions (average temperature 18 ^{o}C).

Plants were harvested 107 days after transplanting. *A. tuberculatus* measurements were taken of height, stem diameter, sex, total above ground and reproductive fresh biomass. Additionally, maize plant measurements were taken of height, stem diameter, total above ground and cob fresh biomass.

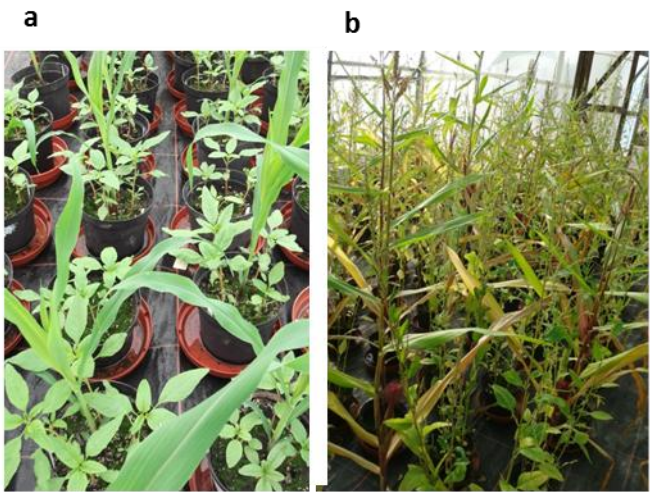


Figure 6.3 Photos from the neighborhood design experiment. a) Multiple pots 48 days after transplanting. **b)** Plants after before harvest 107 days after transplanting.

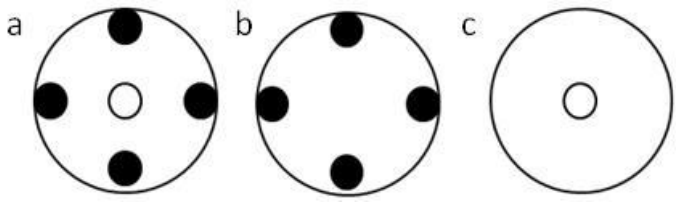


Figure 6.4: Layout of plants in neighbourhood design experiment. Large circles represent pots. Filled circles represent *A. tuberculatus* plants and open small circles represent maize plants. The three treatments allowed the investigation of the impact of *A. tuberculatus* on maize (*a* & *c*) and an indication of the impact of maize on *A. tuberculatus* (*a* & *b*). Layouts *a* & *b* are produced for the six *A. tuberculatus* seed families with contrasting levels of glyphosate resistance. Therefore, layout *a* can determine the relationship between plant measures and seed family resistance levels under inter-specific competition and layout *b* can determine the relationship between *A. tuberculatus* plant measures and seed family resistance levels under inter-specific competition.

6.2.2.1 Statistical Analysis

In order to explore the association between plant measures and seed family LD_{50} a correlation analysis was performed. However, to explore the relationship between seed family gene copy number and plant measures, a regression analysis was performed. Data was analysed in R (R version 2.15.1: 2012-06-22) (R Development Core Team, 2009) regression analysis was performed using the nlme package version 3.1-109 and correlation analysis was performed using the Hmisc package version 3.1-109.

The Shapiro-Wilk test and Bartlett's test were conducted to determine whether data had a normal distribution and homogenous variables respectively. Log or square root transformations were performed if this normalised the distribution and resulted in homogeneous variances between treatments.

A t-test was performed to compare maize biomass in the presence and absence of *A. tuberculatus*. Similarly, a t-test was performed to compare the maize harvest index in the presence and absence of *A. tuberculatus*. Three t-tests were conducted to determine whether the above ground biomass, reproductive biomass and harvest index of *A. tuberculatus* plants were affected by the presence or absence of maize. Finally, an ANOVA was performed to determine whether interspecific competition from maize affects the above ground and reproductive biomass of all seed families equally. All comparative statistics were performed using GenStat (13^{th} Edition).

6.3 Results

6.3.1 Response Surface Design

The response surface data was analysed using a hyperbolic model to determine the relative competitive dynamics of R and S phenotypes (Eq. 6.1, figure 6.5). As a result of competition 7/450 and 16/450 of the S and R plants died, respectively. This mortality was attributed to competition from neighbouring plants, thus, all dead plants were included in the analysis of the response surface data. Predicted biomass in the absence of competition was higher for the S phenotype, relative to the R phenotype (*a* parameter, table 6.2), however, although the magnitude of this difference was great it was not significant (Z_{39} = -0.764, P = 0.22, one tailed). This

lack of significance was attributed to the variation in mean plant biomass between replicates. Additionally, there was no significant difference in the level of the competitive response found in the R and S phenotypes (Table 6.2, Parameter *b*, Z_{39} = -0.717, P = 0.237, one tailed). The susceptible phenotype has a *c* parameter value of 0.48 (± 0.181), this indicates that approximately 0.48 S plants would be required to have an equal competitive effect as an R plant. Conversely, the resistant *c* parameter, with a value of 2.76 (± 0.710), indicates that approximately 2.76 R plants would be required to have an equal competitive effect as an S plant. A comparison of the R & S *c* parameters indicates that the resistant *c* parameter was significantly greater than the susceptible (Z_{39} = 3.107, P < 0.001). Furthermore, the resistant *c* parameter was significantly greater than 1 (t_{39} = 3.89, P < 0.001) and the susceptible *c* parameter was significantly lower than 1 (t_{39} = 2.666, P < 0.05). In summation, the competitive effect of the resistant phenotype on the susceptible phenotype was less than a susceptible plant and the competitive response of a resistant plant was reduced in the presence of a susceptible plant compared to a resistant. Therefore, resistant plants have a lower competitive effect and also a reduced competitive response compared to the susceptible phenotype.

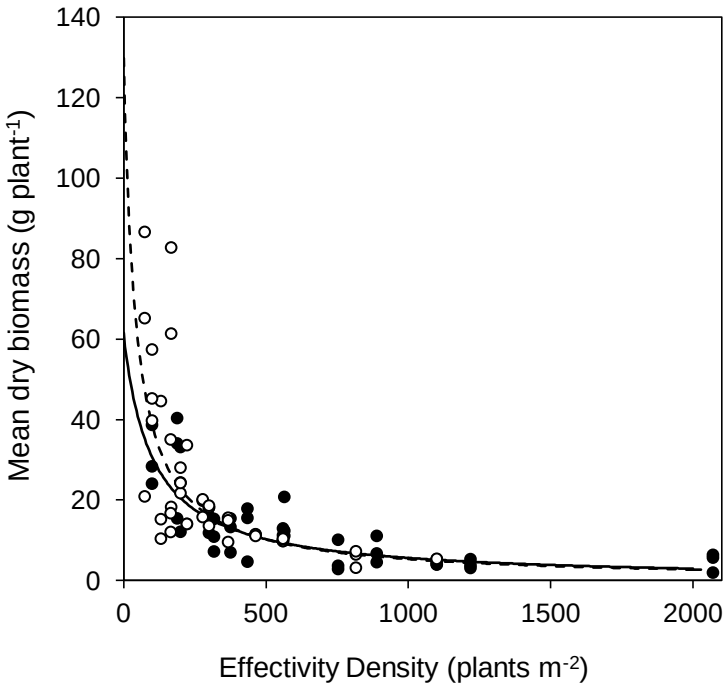


Figure 6.5: Average biomass of R (black) and S (white) phenotypes from each replicate box at each effective density. Analysis includes plants that have died as a result of competition. Data has not been transformed. The solid line is the resistant model and the dashed line is the susceptible model. Effective density is calculated through $N_i + C_{ij}N_j$ where N is the density of the i or j phenotype and C_{ij} is the *c* parameter determined by the either the i or j specific parameter.

Table 6.2: The parameters for the hyperbolic model for resistant (R) and susceptible (S) phenotypes. *a* indicates the maximum yield in the absence of competition; *b* indicates the competitive response of a phenotype to an increase in density and *c* denotes the relative competitive effect of the phenotype. Values in brackets are standard errors of the mean

Phenotype	*a* parameter	*b* parameter	*c* parameter
R	61.5 (30.42)	0.0100 (0.0066)	2.76 (0.710)
S	129.7 (84.02)	0.0235 (0.0177)	0.48 (0.181)

6.3.2 Competitive effect of A. tuberculatus on maize.

The maize impact experiment allows a direct comparison of the competitive effect of *A. tuberculatus* seed families on maize as well as the competitive response of seed families to competition from a single maize plant.

6.3.2.1 A. tuberculatus effect on maize

When the data is pooled across seed family treatments, *A. tuberculatus* plants significantly reduced maize above ground biomass (t_{78}=12.33, P< 0.001), by an average of 42 %. However, the harvest index of maize was not altered by the presence of *A. tuberculatus* competition (t_{78}=-0.07, P= 0.947). There was no significant association between the seed family LD_{50} and the competitive effect of *A. tuberculatus* on maize above ground biomass and harvest index (figure 6.6). Similarly, there was no significant negative relationship between average seed

family *EPSPS* gene copy number and the competitive effect of *A. tuberculatus* on maize above ground biomass and harvest index (figure 6.7).

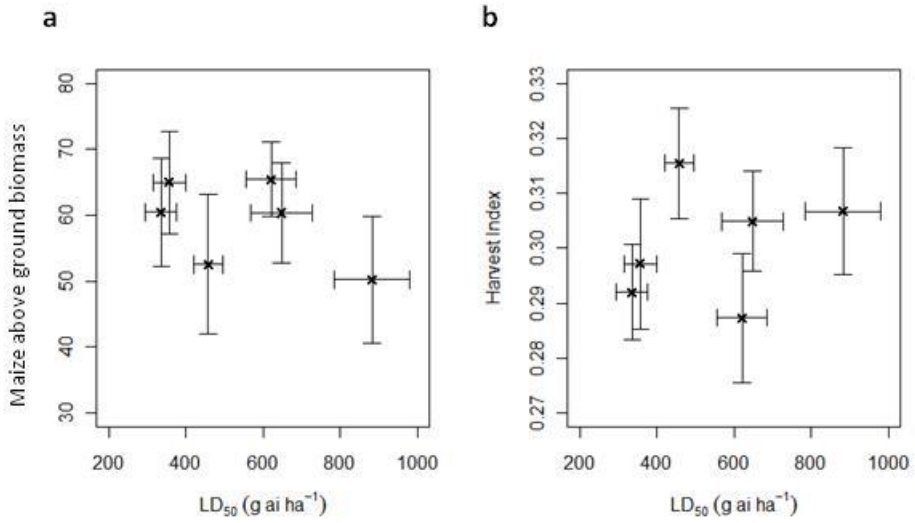


Figure 6.6: a. The relationship between seed family resistance and maize harvest index when grown in the presence of *A. tuberculatus* of competition. P= 0.64, r_4 = 0.25. **b. The relationship between seed family resistance and above ground biomass as a percentage of the control when grown in the presence of *A. tuberculatus* of competition**. P= 0.32, r_4 = -0.49, above ground maize biomass is expressed as a percentage of maize biomass in the absence of *A. tuberculatus* competition. Error bars are standard errors of the mean.

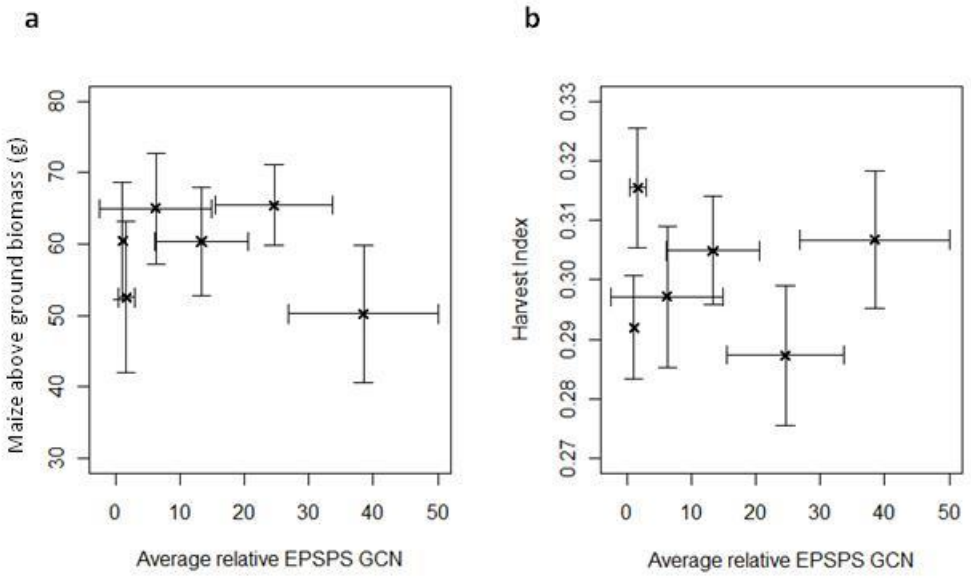


Figure 6.7: a. The competitive effect of *A. tuberculatus* relationship between average seed family relative *EPSPS* gene copy number and maize above ground biomass (a) and harvest index (b) when grown in the presence of *A. tuberculatus*, a. P = 0.59, t_4 = -0.591 **b.** P = 0.92, t_4 = -0.103. Error bars are standard errors of the mean.

6.3.2.2 Effect of Intra-phenotype and maize competition on A. tuberculatus

6.3.2.2.1 Overall impact of maize on A. tuberculatus

Maize plants significantly reduced *A. tuberculatus* above ground biomass (t_{118} = -18.66, P< 0.001, square root transformed data) and reproductive biomass (t_{118} = -8.24, P<0.001, log transformed data) by 37 % and 59 %, respectively. Conversely, the harvest index was increased in the presence of maize competition from 0.19 to 0.29 (t_{118} = 7.44, P < 0.001, log transformed data). Therefore, *A. tuberculatus* plants increase reproductive resource allocation in the presence of maize.

6.3.2.2.1 Seed family relationships

There was no significant negative relationship associated with seed family relative *EPSPS* gene copy number and above ground biomass ($F_{1, 4}$= 0.995, P =0.375) and reproductive biomass ($F_{1, 4}$= 0.174, P = 0.697), when *A. tuberculatus* was grown in the presence of maize (figure 6.8).

There was a significant negative correlation with average seed family relative *EPSPS* gene copy number and above-ground biomass ($F_{1, 4}$= 9.82, P < 0.05) when *A. tuberculatus* was grown in the absence of maize (figure 6.8). The relationship for biomass and *EPSPS* gene copy indicates that 16.6 extra gene copies would cause a 10 % reduction in biomass in the presence of intra-phenotypic competition. Considering that the average gene copy number across all plants tested was 35 gene copies (excluding the extreme individual) it is clear that a growth penalty was associated with amplified gene copy number under intra-specific competition. There was a non-significant negative correlation ($F_{1, 4}$= 6.36, P = 0.065) between gene copy number and reproductive biomass (figure 6.8). Considering the low degrees of freedom associated with this test (4), a repeat experiment with a higher number of seed family experimental units may reveal a significant relationship. There was no discernible relationship between *A. tuberculatus* seed family harvest index and relative *EPSPS* gene copy number under intra-phenotypic competition (figure 6.10).

Similarly, there was a significant negative correlation with average seed family LD_{50} and above ground biomass when *A. tuberculatus* was grown in intra-phenotypic competition in the absence of maize (figure 6.9). Additionally there was a significant negative correlation with average seed family LD_{50} and reproductive biomass but,

there was no significant relationship between harvest index and LD_{50} (figure 6.10). Again there was no relationship between seed family LD_{50} and above ground biomass and reproductive biomass when plants were grown in the presence of maize (figure 6.9). There was no discernible relationship between *A. tuberculatus* seed family harvest index and LD_{50} in the presence of maize or under intra-phenotypic competition (figure 6.10).

In summation, these results indicate that intra-phenotypic competition can increase a growth penalty and produce a fitness cost associated with resistance. Conversely, the inter-specific competition with maize effects all *A. tuberculatus* seed families equally; in terms of above ground biomass ($F_{5,\ 54} = 1.82$, $P < 0.124$) and the reproductive biomass ($F_{5,\ 54} = 1.22$, $P = 0.313$).

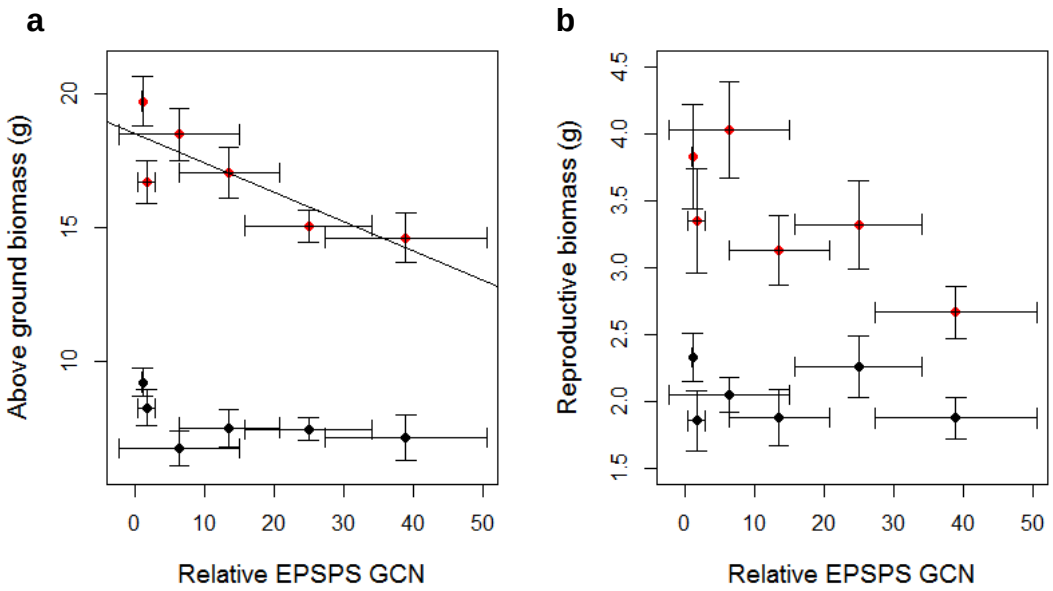


Figure 6.8: The relationship between average relative *EPSPS* gene copy number and *A. tuberculatus* above ground biomass and reproductive biomass. Red points are *A. tuberculatus* measures when plants are grown in intra-phenotypic competition. Black points are *A. tuberculatus* measures when plants are grown in inter-specific competition. Error bars are standard errors of the mean. Solid line indicates significant regression relationship. Regression statistics for **a.** above ground biomass and gene copy number: Inter-specific competition: $F_{1,\ 4}$= 0.995, P =0.375, R^2 = 0.199: Intra-phenotypic competition $F_{1,\ 4}$= 9.82, P < 0.05, y=-0.11x+18.51, R^2 = 0.711, **b.** reproductive biomass and gene copy number: Inter-phenotypic competition: $F_{1,\ 4}$= 0.174, P = 0.697, R^2 = 0.041. Intra-phenotypic competition: $F_{1,\ 4}$= 6.36, P = 0.065, y=-0.026x+3.75, R^2 = 0.614.

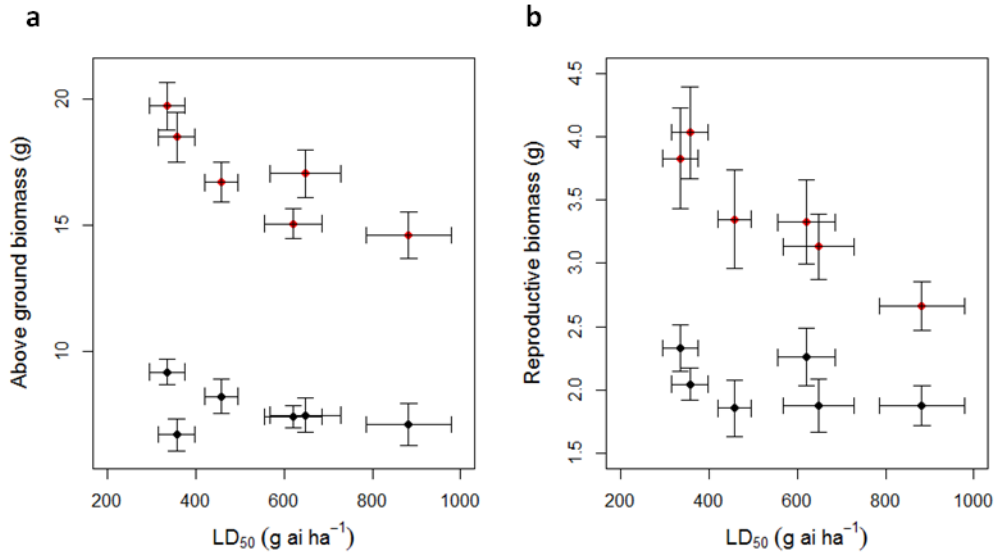


Figure 6.9: The relationship between LD_{50} and *A. tuberculatus* above ground biomass (a) and reproductive biomass (b). Red points are *A. tuberculatus* measures when plants are grown in intra-phenotypic competition. Black points are *A. tuberculatus* measures when plants are grown in inter-specific competition. Error bars are standard errors of the mean. Correlation statistics for **a.** above ground biomass and LD_{50}: Inter-specific competition: r= -0.47, P = 0.375. Intra-phenotypic competition: r= -0.87, P <0.05, **b.** reproductive biomass and LD_{50}: Inter-specific competition: r= -0.47, P = 0.349. Intra-phenotypic competition r= -0.87, P <0.05.

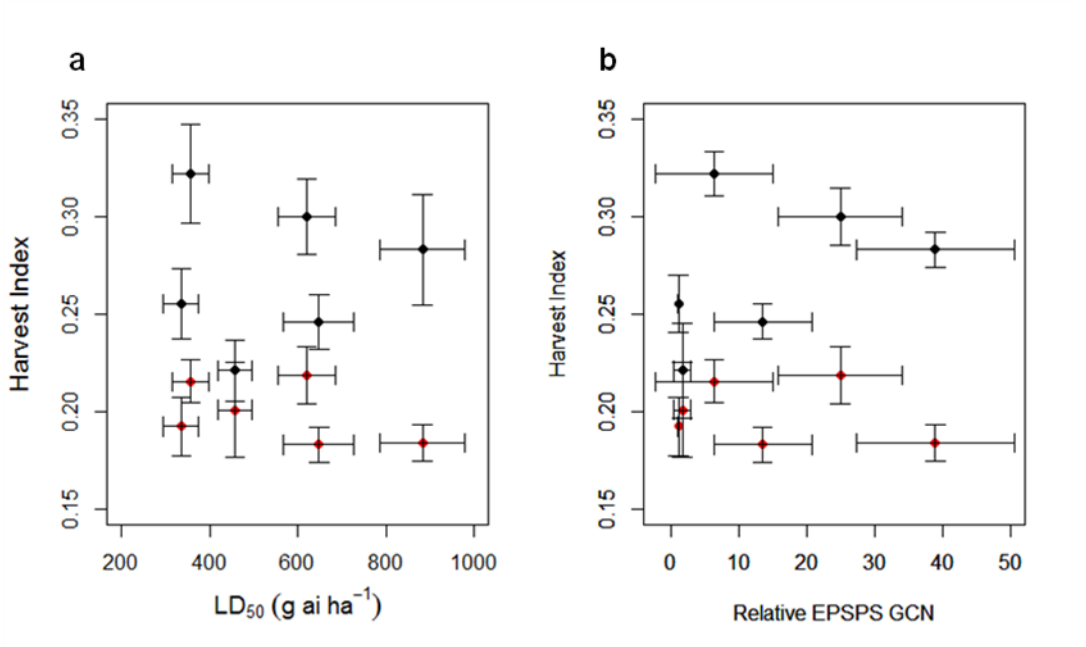


Figure 6.10: a. The relationship between *A. tuberculatus* harvest index and seed family LD_{50} (a) and average relative *EPSPS* gene copy number (GCN) (b) for six seed families. a. Correlation statistics for harvest index and LD_{50}: Inter-specific competition: r= 0.43, P = 0.39. Intra-phenotypic competition: r= 0.55, P = 0.62. **b.** Regression statistics for harvest index and relative *EPSPS* GCN: Inter-specific competition $F_{1, 4}$= 3.092, P = 0.153, R^2 = 0.436. Intra-phenotypic competition $F_{1, 4}$= 0.672, P = 0.458, R^2 = 0.144. Red points are *A. tuberculatus* measures when plants are grown in intra-phenotypic competition. Black points are *A. tuberculatus* measures when plants are grown in inter-specific competition. Error bars are standard errors of the mean.

6.4 Discussion

6.4.1 Response surface experiment

In the response surface experiment there was no significant difference between the predicted biomass of the R and S phenotypes when plants were grown in the absence of competition (parameter *a*) nor the response of phenotypes to competition (parameter *b*). However, the competitive effect of S plants was greater than R plants (parameter *c*). The response surface design measured R and S growth over multiple densities and proportions and as such provide a comprehensive measure of relative plant competition. However, under a natural or agricultural environment weed seed density and phenotype proportion will not be standardised therefore, variation in spatial and local crowding of individuals may influence competitive interactions of R and S plants in a field situation, through the provision of refuge for resistant phenotypes (Weiner *et al.*, 2001; Vila-Aiub *et al.*, 2005)

6.4.1.1 The impact of competition on fitness

Competition can either increase or have no effect on the relative growth of R and S phenotypes (Purrington, 2000). A response surface experiment utilising glyphosate R and S *L. rigidum* found no significant difference in phenotype vegetative growth or competitive ability (Pedersen *et al.*, 2007). However, a metabolically resistant (cytochrome P450) *L. rigidum* individuals exhibited lower vegetative growth which led to a weaker competitive response compared to susceptible individuals (Vila-aiub *et al.*, 2005; Vila-Aiub *et al.*, 2009b).

The impact of competition on triazine resistant and susceptible *A. tuberculatus* was studied in a replacement series design at four different densities. No growth reduction was associated with triazine resistance when resistant and susceptible plants were grown in intra-phenotypic competition. However, S plants were better competitors under inter-specific competition at 50, 100 and 150 plants m^{-2} although no difference was observed at 300 plants m^{-2} (Anderson *et al.*, 1996a).

6.4.1.2 The impact of competition on the EPSPS gene amplification resistance mechanism

A. palmeri population which contains amplified gene copy number did not show a growth penalty or reduction in fitness associated with gene copy number when plants were grown under two different densities (Goh *et al.*, 2013). These results conflict somewhat with the findings from this experiment, however, Goh *et al*.'s experiment was conducted under glasshouse growth conditions and therefore the *A. palmeri* fitness may be studied under sub-optimal conditions to determine the potential for a fitness cost penalty associated with increased gene copy number.

6.4.2 Maize and A. tuberculatus intra- phenotype impact

6.4.2.1 Growth

There was a growth penalty associated with an increase in seed family gene copy number and LD_{50} under intra-phenotypic competition. Specifically, when *A. tuberculatus* plants were grown alongside three neighbouring *A. tuberculatus* plants from the same seed family the total biomass was reduced in relation to seed family resistance level. For example a 10% reduction in plant biomass was associated with

16.6 extra gene copies under intra-phenotypic competition. This intra-phenotypic growth penalty, associated with resistance, was also observed in the polytunnel growth and fitness experiment when plants were grown in the absence of competition (5.3.6). Conversely, the presence of maize mitigated the growth penalty associated with resistance when plants were grown under conditions of intra-phenotypic competition.

6.4.2.2 Fitness

The fitness of *A. tuberculatus* plants was approximated through reproductive biomass. Markedly, there was a fitness trade-off associated with resistance when *A. tuberculatus* plants were grown in intra-phenotypic competition. However, there was no trade-off between *A. tuberculatus* seed family fitness and resistance when plants were grown in inter-specific competition with maize. Furthermore, the competitive effect of maize on *A. tuberculatus* was largely consistent for all seed families. Thus, indicating that maize can mitigate the fitness penalty observed when plants are grown under intra-phenotypic competition.

The fitness of a phenotype is measured in relation to the fitness of the alternative phenotypes, therefore size asymmetric competition can increase the fitness of a highly competitive phenotype within a population when compared to the fitness of a phenotype in the absence of competition (Wall & Begon, 1985; Weiner, 1990). This has occurred in *A. tuberculatus* where the growth penalty observed in the polytunnel experiment has been increased under the presence of intra-phenotypic competition to enhance the relative fitness of the more susceptible phenotypes.

6.4.2.3 Intra and inter-specific competition

Notably, there is a growth and fitness penalty associated with resistance under intra-phenotypic competition but not inter-specific competition with maize. Correspondingly, when *L. rigidum* was grown under low and no crop density, S plants produced more seed than R plants, however, under a high crop density, no difference between R and S seed production was observed (Pedersen *et al.*, 2007). These results indicate that the presence of a crop, particularly at high densities, has the potential to moderate any variation in fitness levels between phenotypes. It may be hypothesized that the crop depleted the available nutrients to produce a stress-inducing low nutrient environment. Indeed, four studies have shown amelioration of a fitness costs under a low nutrient environment (Purrington, 2000). The explanation for this phenomenon was such that a low nutrient environment produced a standard stress response in susceptible individuals, this stress response was equivalent to that exhibited in resistant individuals. A crop fitness mitigating phenomenon may be investigated in alternate species to validate the potential for inter-specific competition to moderate any variation in fitness between the two phenotypes grown under intra-specific competition.

6.4.2.4 Limitations of extrapolating results

The maize and *A. tuberculatus* neighbourhood design experiment does not provide information on weed and crop density or proportion. However, the response surface experiment can determine the effect of weed density and inter-phenotype competition on the relative competitive ability of resistant and susceptible seed

families. Future experiments may look into the impact of maize density and proportion on the growth and fitness of *A. tuberculatus* plants.

6.4.3 Conclusions

There was a negative relationship between *A. tuberculatus* seed family fitness and amplified gene copy when plants were grown in intra-specific competition but not inter-specific competition. Additionally, the intra-phenotypic competition of R on R was lower than the inter-phenotypic competitive effect of S on R. However, the inter-specific competition of S on S was greater than that of R on S. Therefore, the most susceptible *A. tuberculatus* seed family was more competitive than the most resistant seed family when plants were grown across different densities and proportions.

7.0 Discussion

7.1 The resistance epidemic

In 1998, scientists had identified variable glyphosate responses in *A. tuberculatus* field populations (Zelaya & Owen, 2000; Zelaya & Owen, 2002a; Zelaya & Owen, 2005). This was ten years before the first confirmed incidence of glyphosate resistant *Amaranthus tuberculatus* in Missouri (Legleiter & Bradley, 2008)

A major finding from this study was the confirmation of glyphosate resistant populations of *A. tuberculatus* from three sites in Minnesota, USA: Renville, Dumont and Holloway. Resistant indices of 6.6, 5.6 and 4.5 were found for Renville, Dumont and Holloway populations, respectively. The Renville population was collected in 2007 and the Dumont and Holloway populations were collected in 2008. Since these Minnesota populations were initially collected, glyphosate resistant *A. tuberculatus* has established within 14 states of the USA (Heap, 2013). The extent of the resistance epidemic today is the result of the continuous utilisation of glyphosate as a single herbicide system for weed control (Baldwin, 2013). Indeed, the unique selling point of glyphosate tolerant (GT) crops was the provision of a low maintenance one-step weed management strategy. The continued reliance of USA agriculture on GT crops has provided the strong selection pressure required for the evolution of glyphosate resistance (Neve, 2008). This single herbicide system was used until resistance had evolved and established within the field (Baldwin, 2013). The use of glyphosate to control alternate weeds has continued even after resistance has evolved (Beckie, 2011). It is clear from the literature that the knowledge regarding the best management strategies to mitigate resistance establishment is

available (Neve *et al.*, 2003; Neve, 2008; Roux *et al.*, 2008; Neve *et al.*, 2010). As such, the spread of the resistance epidemic may be due to either the ineffective transfer of knowledge to the end user or the lack of implementing the known best practices (Beckie, 2011). Evidently, the communication of knowledge to agrochemical companies, government bodies and most importantly the end user is essential to ensure the implementation of effective resistance management strategies (Vila-Aiub *et al.*, 2008; Beckie, 2011).

7.2 Segregation and inheritance

In order to study the fitness costs associated with glyphosate resistance in *A. tuberculatus*, attempts were made to segregate resistant and susceptible experimental material from the Dumont and Renville populations. Plants with identical phenotypes were crossed to produce resistant and susceptible seed lines. However, a glyphosate response experiment, using a single dose of glyphosate revealed incomplete segregation of the resistance trait in the seed lines when the progeny of parental plants were phenotyped using two segregation methods. Similarly, previous difficulties in segregating the glyphosate resistance trait were found in *A. tuberculatus* populations and in three other weed species (Pratley *et al.*, 1999; Lee & Ngim, 2000; VanGessel, 2001; Zelaya & Owen, 2002; Bell *et al.*, 2009). The incomplete segregation observed in the Dumont and Renville population was attributed to a quantitative mechanism of resistance. Half sibling seed families were maintained as discrete experimental units due to the lack of complete segregation. Thus, the resistance level of 20 seed families was quantified using a dose response analysis. The dose response experiment elucidated a number of seed families

containing discrete LD_{50} values. Further to the elucidation of resistance levels, the parameters of the dose response models could be used to determine the potential for a growth penalty associated with resistance. Indeed, a correlation analysis revealed a negative association between GR_{50} and the weight of plants measured at the control dose (upper asymptote parameters of the dose response model). This trade-off indicated a growth penalty may have been associated with glyphosate resistance in the *A. tuberculatus* Renville and Dumont populations.

7.3 Measuring the fitness of a quantitative trait

The growth and fitness experiments conducted as part of this study utilised a unique experimental method to determine the fitness and growth costs associated with a quantitative resistance trait. The utilisation of half sibling seed families allowed the determination of plant fitness across a spectrum of genotypic and phenotypic resistance levels. A similar method has previously been adopted in the assessment of glyphosate tolerant *I. purpurea* (Baucom & Mauricio, 2004). The seed family method allows fitness measures to be taken of a quantitative resistance trait, where it would be difficult to isolate discrete resistant and susceptible seed lines and where such a categorical assessment would be an oversimplification.

7.4 The mechanism of resistance

The determination of the resistance mechanism can place fitness cost conclusions in context and indicate the potential fitness mechanisms that may produce a fitness cost (Purrington, 2000; Vila-Aiub *et al.*, 2009a). The major mechanism results were the discovery of *EPSPS* gene amplification in the resistant individuals from the Renville

population and the positive relationship between *EPSPS* gene amplification and seed family resistance level. These results indicated that gene amplification was the primary glyphosate resistance mechanism in the Renville population of *A. tuberculatus*. This mechanism has also been found to cause glyphosate resistance in *A. palmeri* (Gaines et al., 2010). The gene amplification of *EPSPS* has been utilised as a molecular marker to screen *A. tuberculatus* populations for glyphosate resistance (Tranel *et al.*, 2011), however, no study has been published to confirm the direct cause of glyphosate resistance in *A. tuberculatus* by *EPSPS* gene amplification (Bell *et al.*, 2009; Tranel *et al.*, 2011; Heap, 2013).

There was some limited evidence that a secondary mechanism may be present within the Renville population. Indeed, an unclassified secondary mechanism of resistance has been proposed in the related species, *A. palmeri*, with the primary resistance mechanism of gene amplification. Indeed, *A. palmeri* plants containing the same gene copy number, displayed different levels of glyphosate resistance when phenotyped using vegetative clones (Sammons, Unpublished). Additionally, two glyphosate resistance mechanisms have been found in a single *A. tuberculatus* population (Tall Waterhemp): target-site alteration and an impaired translocation associated mechanism (Nandula *et al.*, 2013), and three glyphosate resistance mechanisms were found in *Conyza canadensis*, these were impaired translocation, increase *EPSPS* transcript level and altered plant morphology (Dinelli et al., 2006).

Seed family resistance was characterised at the LD_{50} and *EPSPS* gene copy number level. As such, the impact of resistance on plant fitness could be analysed at a phenotypic and genotypic level. It may be hypothesised that the *EPSPS* gene

amplification mechanism could result in a fitness cost due to the energetic drain caused by the EPSPS overproduction (Mauch *et al.*, 2001). Alternatively, if the gene amplification mechanism is mediated by a retro-transposon there is the potential for a fitness penalty to result from the insertion of *EPSPS* gene copies into coding sequences leading to the interruption of essential genes (Mackay, 1989; Gaines *et al.*, 2013).

7.5 Growth and fitness experiments

The impact of a gene amplification mechanism on *A. tuberculatus* fitness was elucidated through a regression analysis on seed family life history traits and average seed family relative *EPSPS* gene copy number (genotypic comparison). However, any possible fitness impact of a potential secondary mechanism of resistance on *A. tuberculatus* fitness was elucidated through the comparison of life history traits with the seed family LD_{50} (phenotypic comparison). Predominantly, the fitness and growth results display trade-offs were observed on a phenotypic and genotypic resistance spectrum. Therefore, *EPSPS* gene copy number was the primary mechanism producing a growth penalty in the Renville population.

No fitness cost was observed in the glasshouse and polytunnel growth and fitness experiments when plants were grown in isolation. When plants from each seed family were grown in the absence of competition, an early growth penalty was associated with glyphosate resistance and *EPSPS* gene copy number under the low light and temperature environment (polytunnel). Conversely a small growth benefit was observed when plants from each seed family were grown in the high light and temperature environment (glasshouse). However, these results are not conclusive and

further experiments must be conducted to determine if a growth benefit associated with resistance within a glass house environment.

The elucidation of a small growth penalty in polytunnel growth conditions led to competition experiments to determine whether this growth penalty could be amplified to produce a fitness cost. Indeed, the competition experiment determined presence of a growth penalty and fitness cost trade-off associated with resistance under intra-phenotypic competition. However, the interspecific competition from maize alongside intra-phenotypic competition from *A. tuberculatus* mitigated this resistance cost as maize reduced the growth of plants from all seed families equally.

7.6 Environmental conditions

The glasshouse experiment had an average temperature of 21 ^{o}C whereas the polytunnel has an average temperature of 18 ^{o}C. Furthermore, the glasshouse contained additional lighting whereas the polytunnel did not. Therefore, it is likely that the light and temperature levels in polytunnel experiments have influenced the growth and fitness of *A. tuberculatus* plants as no supplementary light was provided in the polytunnel experiment. The lower light and temperature growth conditions in the polytunnel experiment resulted in a reduced plant relative growth rate compared to the glasshouse; this confirmed that suboptimal growth conditions were experienced in the polytunnel experiment.

Further to the alteration in abiotic conditions, two different soil media were utilised in the polytunnel and glasshouse experiments. This additional experimental variation somewhat confounds the potential to conclude that the abiotic differences between

the two environments have caused the alteration in fitness observations. As low resource availability can influence the expression of a growth penalty. A growth penalty may even be mitigated by the low nutrient soil medium (Purrington, 2000). However, the competition experiment was conducted under a low light environment using low nutrient growth medium (the same utilised in the glasshouse growth and fitness experiments), therefore, the elucidation of a growth penalty, all be it under conditions of intra-phenotypic competition, indicates that the low nutrient growth medium has less influence on the fitness penalty than the growth environment.

7.7 Theory of costs of adaptation

In spite of the variation associated with the fitness data, the conclusions from this study support the theory of the costs of adaptation, alongside an expanding body of evidence that some herbicide resistance alleles are associated with penalties (Bergelson & Purrington, 1996; Vila-Aiub *et al.*, 2009a). The results from this study show that no penalty was associated with the resistance alleles under high temperatures and light growth conditions. However, there was a small growth penalty observed under low light and temperature growth conditions. Finally there was a fitness penalty associated with resistance when plants were grown under intra-phenotypic competition; however, this cost was mitigated under inter-specific competition from maize. A meta-analysis conducted on anti-herbivory plant fitness costs determined that costs were less likely to be observed in controlled as oppose to uncontrolled environmental conditions, as observed in the glasshouse versus polytunnel experiments (Koricheva, 2002). Similarly, triazine resistant fitness costs may be enhance or decreased by alteration in light and temperature levels (Vila-Aiub

et al., 2009a). Furthermore, this study found that a growth penalty was increased to produce a fitness cost under intra-specific competition. Similarly intra-specific interactions were found to elucidate fitness cost in *Lollium rigidum* and *Nicotiana attenuata* (Van Dam & Baldwin, 2001; Pedersen *et al.*, 2007).

It is generally accepted that biotic and abiotic environments impact the expression of a cost (Koricheva, 2002; Vila-Aiub *et al.*, 2009a). Indeed, the findings from this study indicate that environmental and competitive interactions can determine the potential for elucidation of a fitness cost. The results from this study confirm that both biotic and abiotic interactions may determine whether the resistance cost may be exhibited.

7.8 Mitigation of the glyphosate resistant *A. tuberculatus* epidemic

Generally, increasing the complexity of weed management strategies can help to mitigate the evolution of resistance (Owen *et al.*, 2013). Increasing the number of strategies provides multiple selection pressures for weeds to resist and therefore a lower potential for individuals to evolve resistance to all forms of control. A five year study was conducted to determine the efficacy of adding non-glyphosate weed management strategies to GT crop production regimes over six American states. The addition of alternate weed management strategies, mitigated or reduced resistant weed species compared to a glyphosate-only system and glyphosate susceptibility was maintained in non-resistant weed populations. However, it took five years before the benefits of the additional weed management strategies were observed (Owen *et al.*, 2013).

In response to the considerable *A. palmeri* glyphosate resistance epidemic, multiple resistance management strategies have been adopted in Southern USA states (Norsworthy, 2013). Increasing the complexity of *A. tuberculatus* management strategies in GT crop regimes using similar methods to those adopted for *A. palmeri* may mitigate the risk of resistance. Such strategies include hand weeding of uncontrolled weeds and reduced crop row spacing (Norsworthy, 2013). When soils do not require the implementation of conservation tillage to preserve soil properties (Kemper & Derpsch, 1981; Reeves, 1997), tillage remains an effective control strategy for *A. tuberculatus* (Steckel *et al.*, 2007). Furthermore, herbicide based control tactics are often utilised to mitigate resistant weeds, these include the application of alternative herbicidal modes-of-action; either in sequence or in mixtures and switching to alternate mode-of-action resistance crops (Beckie, 2011). Also, the use of pre-emergence herbicide applications which can kill germinating seedlings and thus provide an alternate control tactic (Beckie, 2011; Norsworthy, 2013).

7.9 Implications of results- weed control potential

7.9.1 Fitness informed management

More specifically than increasing the general complexity of weed management, strategies may be targeted to manipulate the pleiotropic fitness costs associated with resistance. Such strategies may be utilised to prevent resistance evolution or aid the control of existing resistant populations (Vila-aiub *et al.*, 2005). Weed control tactics may enhance discrepancies in life history traits and enhance penalties to produce

fitness cost associated with resistant plants. The targeting of weed management strategies could decrease the fitness of any arising glyphosate resistant *A. tuberculatus* plants and thus reduce the potential for resistance to establish within a population.

The discrepancy between the glasshouse and polytunnel fitness results suggests a genotype-by-environment interaction with fitness. It may be possible to enhance the small growth penalty associated with glyphosate resistance by manipulating growth conditions to produce a low light environment. This could be achieved through the use of a cover crop, or through decreasing crop row spacing. Indeed, decreasing row spacing of maize reduces photosynthetically active radiation below the crop canopy (Tharp & Kells, 2001), thus providing lower light conditions to promote the enhancement of a resistance growth penalty. Furthermore, the use of a cover crop or decreased row spacing can have additional benefits through enhancing phtyochrome regulated *A. tuberculatus* seed dormancy universally, by decreasing the ratio of red: far red light reaching the seed bank (Leon & Owen, 2003). Field trials are essential to test the hypothesis that producing low-light abiotic conditions will lead to a growth penalty, particularly because the inter-specific competition from maize was found to mitigate discrepancies observed between seed family fitness.

The presence of a resistance cost under intra-phenotypic competition and the higher competitive ability of the most susceptible seed family indicates that resistant *A. tuberculatus* individuals are likely to have a lower relative fitness in the field. Consequently, the frequency of resistance alleles would reduce within the field if the Renville population was not exposed to glyphosate application and after numerous

generations' glyphosate susceptibility may be restored to populations. This study provides a basis upon which future research may be conducted in order to determine the best resistance management strategies within the field.

7.9.2 Prediction of resistance evolution using models

Fitness parameters are required to predict the frequency of resistance evolution under specified management strategies. For example, a predictive model was produced to study glyphosate resistance evolution in weed populations under a GM crop production regime (Neve, 2008). The model confirms that a 20 % reduction in relative fitness was associated with a monogenic mechanism of resistance and a dominant fitness cost, can delay the onset of resistance. Specifically, the presence of a cost would reduce the number of populations evolving resistance from a 26 to 11% where five glyphosate applications are made under a GT crop production system (Neve, 2008). Moreover, species specific models may be produced to investigate the impact of management strategies on the evolution of glyphosate resistance. For example, models have been produced to investigate the impact of resistance management strategies in *Amaranthus palmeri*, (Neve *et al.*, 2010; Neve *et al.*, 2011) however, the model does not currently incorporate the fitness costs associated with resistance. The analysis of *A. tuberculatus* Renville population under a competitive environment will produce fitness parameters that may be included in a similar predictive simulation model. The model may be utilised to aid the prediction of glyphosate resistance evolution in *A. tuberculatus* and thus determine the risk of glyphosate resistance evolution under different management strategies. Such a model

can determine whether the frequency of the resistance alleles will decrease in the population over time under no selection.

7.10 Conclusions

- Glyphosate resistance was found in three populations of *A. tuberculatus* from Minnesota.
- The incomplete segregation of resistant and susceptible seed lines indicated that resistance was controlled by a quantitative resistance trait in Renville and Dumont populations.
- *EPSPS* gene amplification was the primary mechanism of glyphosate resistance in the *A. tuberculatus* Renville population.
- A negative association was observed between seed family GR_{50} and the upper asymptote of the dose response models for the 20 seed families.
- There was no fitness trade-off associated with Renville seed family resistance or gene copy number when plants are grown in isolation, in polytunnel and glasshouse growth environments.
- There was an small growth trade-off associated with seed family resistance and gene copy number when plants were grown in the absence of competition in polytunnel but not glasshouse growth environments.
- There was a growth trade-off associated with seed family resistance and gene copy number when plants were grown in the absence of competition such that a 10% reduction in plant biomass was associated with 21 extra gene copies.
- A substitution rate of 2.76 was determined for *A. tuberculatus* individuals from most resistant seed family whereas a substitution rate of 0.48 was

determined for the most susceptible seed family. These values indicate that the resistant individuals had weaker competitive effect and response than the susceptible individuals.

- There was a growth trade-off associated with seed family resistance and gene copy number when grown under intra-phenotypic competition such that a 10% reduction in plant biomass was associated with 16.6 extra gene copies.
- Inter-specific competition from maize mitigated the growth and fitness trade-off associated with resistance under intra-phenotypic competition.

7.11 Suggested future work

7.11.1 Resistance mechanism

The mechanism of gene amplification may impact on the segregation of plant R and S seed families and may influence the potential fitness cost mechanisms. A substantial body of work may be conducted to determine the genetic mechanism that results in gene amplification in *A. turberculatus* (Gaines et al., 2013). However, the genetic mechanism of gene amplification has not yet been elucidated within the related species *A. palmeri* (Gaines *et al.*, 2013).

The un-sequenced portion *EPSPS* gene may be sequenced to determine the presence of a mutation in the remaining of the *EPSPS* sequence. However, no such resistance endowing mutations have been reported thus far within the literature and glyphosate binds specifically at the PEP binding site of EPSPS (Schönbrunn *et al.*, 2001).

Therefore, it is unlikely that a non-binding site specific mutation will evolve to alter the glyphosate/ PEP binding site whilst preserving function and causing resistance.

17.11.2 Fitness

Future work should also look at the influence of genetic background on the resistance costs. The genetic background of a population impacts the expression of a fitness cost. A 2,4-D target-site resistance mutation was studied in nine genetic backgrounds of *A. thaliana* to reveal that fitness (for example, number of siliques) and quantitative traits (for example rosette diameter) differed amongst genetic backgrounds (Paris *et al.*, 2008). Therefore, it is important to study the impact of the *EPSPS* gene amplification mechanism across multiple *A. tuberculatus* populations to determine if the observed growth and fitness penalties are present across multiple genetic backgrounds.

To provide a comprehensive analysis of the impact of amplified *EPSPS* gene copy number on *A. tuberculatus* fitness, future experiments should investigate fitness over more life history stages including discrepancies in seed number, pollen viability and seed viability (Vila-Aiub *et al.*, 2009a). The current study has used flower biomass to indicate the relative fitness of seed families for two reasons. Primarily, a large volume of seed can be produced by *A. tuberculatus* plants (up to 2000000 seeds plant^{-1} (Battles *et al.*, 1998) which not only requires a substantial investment of time to harvest and clean-seed, but also the development of a method to retain fallen mature seed (a logistically difficult task). Secondly, total seed production and viability is a function of both pollen and ovule frequency and viability. Inconveniently, *A. tuberculatus* is a dioecious species; therefore, plants cannot self-

fertilise. Consequently, to produce seed with a discrete phenotype for fitness analysis, identical phenotypes must be crossed in isolation. However, for a quantitative resistance mechanism, male and female parental plants will both contain a distinct resistance level and thus the resulting seed produced will contain a resistance level and thus also a fitness level which is a product of the two phenotypes. Furthermore, the sex of plants cannot be determined before the point of flowering, therefore, identical phenotype crosses cannot be set up in a controlled manner before the reproductive phase of plants has occurred. Even if segregation crosses were conducted the selected plants would need to be grown in isolation, therefore environmental variation may confound fitness measures.

17.11.3. Competition

Crop proportion and density may influence the competitive effect and response of *A. tuberculatus* seed families containing different levels of glyphosate resistance. Therefore, a response surface design experiment with maize and *A. tuberculatus* could confirm that there is no relationship between *EPSPS* gene copy number and competitive interactions with maize. Furthermore, the competitive interactions across multiple *A. tuberculatus* seed families may be measured using a response surface design experiment.

17.11.4 Allele frequency experiment

The true measure of a fitness cost may be determined through allele frequency after multiple generations in the absence of selection. Ideally, an allele frequency experiments should be conducted within the field and under competition from other

plants. Such conditions are particularly advisable due to the observed influence of biotic and abiotic factors on growth and fitness in the above experiments. Therefore, given unlimited experimental resources and assuming no ethical concerns (such as the release of resistance traits into the environment), resistance trait frequency may be measured over an experimental cline to determine the fitness cost associated with the resistance trait.

8.0 References

Alarcon-Reverte, R., Garci, A., Jasieniuk, M., Lanini, T., Hanson, B. D. & Fischer, A. J. (2011) *Glyphosate Driven Selection Strikes Again: Investigating the Mechanism of Resistance in Echinochloa colona from California*. IN: Weed Science Society of America Abstract (2011).

Anderson, D. D., Higley, L. G., Martin, A. R. & Roeth, F. W. (1996a) Competition between triazine-resistant and -susceptible common waterhemp (Amaranthus rudis). *Weed Science*, 44 (4): 853-859.

A**nderson, D. D., Roeth, F. W. & Martin, A. R.** (1996b) Occurrence and control of triazine-resistant common waterhemp (Amaranthus rudis) in field corn (Zea mays). *Weed Technology*, 10 (3): 570-575.

Annou, M., Thomsen, M. & Wailes, E. (2001) Impacts of herbicide resistant rice technology on rice-soybeans rotation. *AgBioForum*, 4 (2 Cited April 28, 2003): 79-86.

Baerson, S. R., Rodriguez, D. J., Tran, M., Feng, Y., Biest, N. A. & Dill, G. M. (2002) Glyphosate-Resistant Goosegrass. Identification of a Mutation in the Target Enzyme 5-Enolpyruvylshikimate-3- Phosphate Synthase. *Plant Physiology*, 129 1265-1275.

Baldwin, F. (2013) *What a difference 10 years can make!* IN: Global herbicide resistance challenge conference. Austrlian herbicide resistance initiative, Perth, Australia. pp. 30.

Balmford, A., Green, R. & Phalan, B. (2012) What conservationists need to know about farming. *Proceedings. Biological sciences / The Royal Society*, 2714-2724.

Banik, P., Midya, A., Sarkar, B. K. & Ghose, S. S. (2006) Wheat and chickpea intercropping systems in an additive series experiment: Advantages and weed smothering. *European Journal of Agronomy*, 24 (4): 325-332.

Bates, D. M. & Watts, D. G. (1988) *Nonlinear Regression Analysis and Its Applications,*. New York: Wiley & Sons

Battaglin, W. A., Kolpin, D. W., Scribner, E. A., Kuivila, K. M. & Sandstrom, M. W. (2005) Glyphosate, other herbicides, and transformation products in Midwestern streams, 2002. *Journal of the American Water Resources Association*, 41 (2): 323-332.

Battles, B., Hartzler, B. & Buhler, D. (1998) Effect of common waterhemp emergence date in soybeans on growth and competitiveness. Proc. *Proc. N. Cent. Weed Sci. Soc.*, 53 145–146.

Baucom, R. S. & Mauricio, R. (2004) Fitness costs and benefits of novel herbicide tolerance in a noxious weed. *Proceedings of the National Academy of Sciences of the United States of America*, 101 (36): 13386-13390.

Baylis, A. D. (2000) Why glyphosate is a global herbicide: strengths, weaknesses and prospects. *Pest management science*, 56 299-308.

Beckie, H. J. (2006) Herbicide-resistant weeds: Management tactics and practices. *Weed Technology*, 20 (3): 793-814.

Beckie, H. J. (2011) Herbicide-resistant weed management: focus on glyphosate. *Pest management science*, 67 1037-1048.

Beckie, H. J. & Tardif, F. J. (2012) Herbicide cross resistance in weeds. *Crop Protection*, 35 15-28.

Bell, M. S. & Tranel, P. J. (2010) Time Requirement from Pollination to Seed Maturity in Waterhemp (Amaranthus tuberculatus). *Weed Science*, 58 (2): 167-173.

Bell, M. S., Tranel, P. J. & Riggins, C. W. (2009) Glyphosate resistance in waterhemp: inheritance and EPSPS copy number. *North Central Weed Sci. Soc. Abstr.*, 64: 100

Benachour, N. & Séralini, G. E. (2009) Glyphosate formulations induce apoptosis and necrosis in human umbilical, embryonic, and placental cells. *Chemical research in toxicology*, 22 (1): 97-105.

Bensch, C. N., Horak, M. J. & Peterson, D. (2003) Interference of redroot pigweed (Amaranthus retroflexus), Palmer amaranth (A. palmeri), and common waterhemp (A. rudis) in soybean. *Weed Science*, 51 (1): 37-43.

Bentley, W. E., Mirjalili, N., Andersen, D. C., Davis, R. H. & Kompala, D. S. (1990) Plasmid encoded protein the principal factor in the metabolic burden associated with recombinant bacteria. *Biotechnology and Bioengineering*, 35 (7): 668-681.

Bergelson, J. & Purrington, C. B. (1996) Surveying patterns in the cost of resistance in plants. *The American Naturalist*, 148 (3): 536-558.

Boocock, M. R. & Coggins, J. R. (1983) Kinetics of 5-enolpyruvylshikimate-3-phosphate synthase inhibition by glyphosate. *Febs Letters*, 154 (1): 127-133.

Bradshaw, L. D., Padgette, S. R., Kimball, S. L. & Wells, B. H. (1997) Perspectives on glyphosate resistance. *Weed Technology*, 11 (1): 198-198

Bridges, D. C. (1994) Impact of Weeds on Human Endeavors. *Weed Technology*, 8 (2): 392-395.

Buhler, D. D. (1992) Population-dynamics and control of annual weeds in corn (*Zea mays*) as influenced by tillage systems. *Weed Science*, 40 (2): 241-248.

Busi, R., Yu, Q., Barrett-Lennard, R. & Powles, S. (2008) Long distance pollen-mediated flow of herbicide resistance genes in Lolium rigidum. *Theoretical and Applied Genetics*, 117 (8): 1281-1290.

Causton, D. & Venus, J. (1981) *The biometry of plant growth*. London, UK: Edward Arnold.

Choudhuri, S., Cui, Y. & Klaassen, C. D. (2010) Molecular targets of epigenetic regulation and effectors of environmental influences. *Toxicology and Applied Pharmacology*, 245 (3): 378-393.

Cohan, F. M., King, E. C. & Zawadzki, P. (1994) Amelioration of the deleterious pleiotropic effects of an adaptive mutation in *Bacillus subtilis*. *Evolution*, 48 (1): 81-95.

Cole, D. J. (1985) *Mode of action of glyphosate- a literature analysis, in the herbicide glyphosate*. London: Butterworths.

Collavo, A. & Sattin, M. (2012) Resistance to glyphosate in Lolium rigidum selected in Italian perennial crops: bioevaluation, management and molecular bases of target-site resistance. *Weed Research*, 52 (1): 16-24.

Cousens, R. (1991) Aspects of the design and interpretation of competition (interference) experiments. *Weed Technology*, 5 (3): 664-673.

Cousens, R. & Mortimer, A. (1995) *Dynamics of weed populations*. Cambridge: Cambridge University Press

Cousens, R. D., Gill, G. S. & Speijers, E. J. (1997) Comment: Number of sample populations required to determine the effects of herbicide resistance on plant growth and fitness. *Weed Research*, 37 (1): 1-4.

Coustau, C., Chevillon, C. & Ffrench-Constant, R. (2000) Resistance to xenobiotics and parasites: can we count the cost? *Trends in ecology & evolution (Personal edition)*, 15 (9): 378-383.

Culpepper, A. S., Grey, T. L., Vencill, W. K., Kichler, J. M., Webster, T. M., Brown, S. M., York, A. C., Davis, J. W. & Hanna, W. W. (2006) Glyphosate-resistant Palmer amaranth (Amaranthus palmeri) confirmed in Georgia. *Weed Science*, 54 (4): 620-626.

Darmency, H. (2013) Pleiotropic effects of herbicide-resistance genes on crop yield: a review. *Pest management science*, 69 (8): 897-904.

Darwin, C. (1859) *The Origin of Species*. J. Murray: London.

de Carvalho, L. B., da Costa Aguiar Alves, P. L., Gonzalez-Torralva, F., Enrique Cruz-Hipolito, H., Maria Rojano-Delgado, A., De Prado, R., Gil-Humanes, J., Barro, F. & Luque de Castro, M. D. (2012) Pool of Resistance Mechanisms to Glyphosate in Digitaria insularis. *Journal of Agricultural and Food Chemistry*, 60 (2): 615-622.

Dekker & Duke (1995) Herbicide-Resistant Field crops. *Advances in Agronomy*, 54: 69-116.

Dill, G. M., CaJacob, C. A. & Padgette, S. R. (2008) Glyphosate-resistant crops: adoption, use and future considerations. *Pest Management Science*, 64 (4): 326-331.

Dinelli, G., Marotti, I., Bonetti, A., Catizone, P., Urbano, J. M. & Barnes, J. (2008) Physiological and molecular bases of glyphosate resistance in Conyza bonariensis biotypes from Spain. *Weed Research*, 48 (3): 257-265.

Dinelli, G., Marotti, I., Bonetti, A., Minelli, M., Catizone, P. & Barnes, J. (2006) Physiological and molecular insight on the mechanisms of resistance to glyphosate in Conyza canadensis (L.) Cronq. biotypes. *Pesticide Biochemistry and Physiology*, 86 (1): 30-41.

Duke, S. O. (2011) Glyphosate Degradation in Glyphosate-Resistant and -Susceptible Crops and Weeds. *Journal of Agricultural and Food Chemistry*, 59 (11): 5835-5841.

Duke, S. O. (2012) Why have no new herbicide modes of action appeared in recent years? *Pest Management Science*, 68 (4): 505-512.

Duke, S. O. & Powles, S. B. (2008) Glyphosate: a once-in-a-century herbicide. *Pest Management Science*, 64 (4): 319-325.

Dyer, W. E. (1994) *Herbicide Resistance in Plants: Biology and Biochemistry*. BocaRaton/Ann Arbor/London/Tokyo: Lewis Publ.

Eschenburg, S., Healy, M. L., Priestman, M. A., Lushington, G. H. & Schonbrunn, E. (2002) How the mutation glycine96 to alanine confers glyphosate insensitivity to 5-enolpyruvyl shikimate-3-phosphate synthase from Escherichia coli. *Planta*, 216 (1): 129-135.

FAO, Food and Agriculture Organization of the United Nations. (2009) *State of Food Insecurity in the World 2009.* Rome, Italy: FAO. [online] Available from: ftp://ftp.fao.org/docrep/fao/012/i0876e/i0876e.pdf (**Acessed August 30, 2013**).

Feng, P. C. C., Tran, M., Chiu, T., Sammons, R. D., Heck, G. R. & CaJacob, C. A. (2004) Investigations into glyphosate-resistant horseweed (Conyza canadensis): retention, uptake, translocation, and metabolism. *Weed Science*, 52 (4): 498-505.

Fisher, R. A. (1928) The possible modification of the responses of wild type to recurrent mutations. *American Naturalist*, 62: 115-126.

Foresman, C. & Glasgow, L. (2008) US grower perceptions and experiences with glyphosate-resistant weeds. *Pest Management Science*, 64 (4): 388-391.

Foster, S. P., Young, S., Williamson, M. S., Duce, I., Denholm, I. & Devine, G. J. (2003) Analogous pleiotropic effects of insecticide resistance genotypes in peach-potato aphids and houseflies. *Heredity*, 91 (2): 98-106.

Franssen, A. S., Skinner, D. Z., Al-Khatib, K., Horak, M. J. & Kulakow, P. A. (2001) Interspecific hybridization and gene flow of ALS resistance in Amaranthus species. *Weed Science*, 49 (5): 598-606.

Franz, J., Mao, M. & Sikorski, J. (1997) *Glyphosate: A Unique Global Herbicide*. Washington DC: American Chemical Society. 521-615.

Fry, J. D. (2003) Detecting ecological trade-offs using selection experiments. *Ecology*, 84 (7): 1672-1678.

Funke, T., Yang, Y., Han, H., Healy-Fried, M., Olesen, S., Becker, A. & Schoenbrunn, E. (2009) Structural Basis of Glyphosate Resistance Resulting from the Double Mutation Thr(97) -> Ile and Pro(101) -> Ser in 5-Enolpyruvylshikimate-3-phosphate Synthase from Escherichia coli. *Journal of Biological Chemistry*, 284 (15): 9854-9860.

Gaines, T., Ward, S. M., Bukun, B., Preston, C., Leach, J. E. & Westra, P. (2011) Interspecific hybridization transfers a previously unknown glyphosate resistance mechanism in Amaranthus species. *Evolutionary Applications*, 5 (1): 29-38.

Gaines, T., Zhang, W., Wang, D., Bukun, B., Chisholm, S. T., Shaner, D. L., Nissen, S. J., Patzoldt, W. L., Tranel, P. J., Culpepper, a. S., Grey, T. L., Webster, T. M., Vencill, W. K., Sammons, R. D., Jiang, J., Preston, C., Leach, J. E. & Westra, P. (2010) Gene amplification confers glyphosate resistance in Amaranthus palmeri. *Proceedings of the National Academy of Sciences of the United States of America*, 107 (3): 1029-1034.

Gaines, T. A., Ward, S. M., Bukun, B., Preston, C., Leach, J. E. & Westra, P. (2012) Interspecific hybridization transfers a previously unknown glyphosate resistance mechanism in Amaranthus species. *Evolutionary Applications*, 5 (1): 29-38.

Gaines TA, Wright AA, Molin WT, Lorentz L, Riggins CW, et al. (2013) Identification of Genetic Elements Associated with *EPSPS* Gene Amplification. *PLoS ONE* 8(6): e65819. doi:10.1371/journal.pone.0065819

Gaines, T. A., Wright, A. A., Molin, W. T., Lorentz, L., Riggins, C. W., Tranel, P. J., Beffa, R., Westra, P. & Powles, S. B. (2013) *Miniature inverted-repeat transposable elements (MITEs) associated with EPSPS gene amplification in Amaranthus palmeri*. IN: Australian Herbicide Resistance Initiative. Global Herbicide Resistance Challenge conference, 2013 Perth. Australia pp. 38.

Gardner, S. N., Gressel, J. & Mangel, M. (1998) A revolving dose strategy to delay the evolution of both quantitative vs major monogene resistances to pesticides and drugs. *International Journal of Pest Management*, 44 (3): 161-180.

Gassmann, A. J. & Futuyma, D. J. (2005) Consequence of herbivory for the fitness cost of herbicide resistance: photosynthetic variation in the context of plant-herbivore interactions. *Journal of Evolutionary Biology*, 18 (2): 447-454.

Ge, X., d'Avignon, D. A., Ackerman, J. J. & Sammons, R. D. (2010) Rapid vacuolar sequestration: the horseweed glyphosate resistance mechanism. *Pest management science*, 66 (4): 345-348.

Ghersa, C. M., Benech-Arnold, R. L., Satorre, E. H. & Matinez-Ghersa, M. A. (2000) Advances in weed management strategies. *Field Crops Research*, 67 (2): 95-104.

Ghersa, C. M., Roush, M. L., Radosevich, S. R. & Cordray, S. M. (1994) Coevolution of argoecosystems and weed management. *BioScience*, 44 85-94.

Gianessi, L. P. (2005) Economic and herbicide use impacts of glyphosate-resistant crops. *Pest Management Science*, 61 (3): 241-245.

Gianessi, L. P. & Reigner, N. P. (2007) The Value of Herbicides in U.S. Crop Production. *Weed Technology*, 21 (2): 559-566.

Goh, S. S., Han, H., Yu, Q., Busi, R., Vila-Aiub, M., Gaines, T. & Powles, S. (2013) *Glyphosate resistance endowed by EPSPS gene amplification does not attract a fitness cost in Amaranthus palmeri*. IN: Global Herbicide Resistance Challenge 2013. Perth, Australia. Australian Herbicide Resistance Initiative. pp. 87.

Goldberg, D. E. (1990) *Components of resource competition in plant communities*. San Diego: Academic Press.

Goldberg, D. E. & Werner, P. A. (1983) Equivalence of competitors in plant communities a null hypothesis and a field experimental approach. *American Journal of Botany*, 70 (7): 1098-1104.

Hager, A. G., Wax, L. M., Bollero, G. a. & Simmons, F. W. (2002) Common waterhemp (Amaranthus rudis Sauer) management with soil-applied herbicides in soybean (Glycine max (L.) Merr.). *Crop Protection*, 21 (4): 277-283.

Harms, C. T., Armour, S. L., Dimaio, J. J., Middlesteadt, L. A., Murray, D., Negrotto, D. V., Thompsontaylor, H., Weymann, K., Montoya, A. L., Shillito, R. D. & Jen, G. C. (1992) Herbicide resistance due to amplification of a mutant acetohydroxy acid synthase gene. *Molecular & General Genetics*, 233 (3): 427-435.

Hartzler, R. (2003) *Waterhemp—The perfect weed?* Weed Science Society of America [online] Available from:http://web.archive.org/web/20061018184807/http://www.weeds.iastate.edu/mgmt/2003/symposium.shtml (**Accessed 31 May, 2013**).

Hartzler, R. G., Buhler, D. D. & Stoltenberg, D. E. (1999) Emergence characteristics of four annual weed species. *Weed Science*, 47 (5): 578.584.

Hausman, N. E., Singh, S., Tranel, P. J., Riechers, D. E., Kaundun, S. S., Polge, N. D., Thomas, D. A. & Hager, A. G. (2011) Resistance to HPPD-inhibiting herbicides in a population of waterhemp (*Amaranthus tuberculatus*) from Illinois, United States. *Pest Management Science*, 67 (3): 258-261.

Heap, I. (2005) Initial Characterization of Resistance vs. Routine Screening.In: *Criteria for Confirmation of Herbicide-Resistant Weeds* [online] Available from:http://www.weedscience.org/Documents/ResistanceCriterion.pdf (Accessed **August 23, 2013**).

Heap, I. (2006) *Glyphosate-resistant Weeds: A Global Perspective.* Illinois Crop Protection, Technology Conference. University of Illinois Extension 36-40.

Heap, I. (2013) *The International Survey of Herbicide Resistant Weeds.* [online] Available from: www.weedscience.com (Accessed **August 23, 2013**).

Heap, I. & LeBaron, H. (2001) Introduction and Overview of Resistance. In: *Herbicide Resistance and World Grains*. CRC Press LLC, Florida pp. 3

Heil, M. & Baldwin, I. T. (2002) Fitness costs of induced resistance: emerging experimental support for a slippery concept. *Trends in plant science*, 7 (2): 61-67.

Herms, D. A. & Mattson, W. J. (1992) The dilemma of plants - to grow or defend. *Quarterly Review of Biology*, 67 (3): 283-335.

Hoffmann, W. A. & Poorter, H. (2002) Avoiding bias in calculations of relative growth rate. *Annals of Botany*, 90 (1): 37-42.

Horak, M. J. & Loughin, T. M. (2000) Growth analysis of four Amaranthus species. *Weed Science*, 48 (3): 347-355.

Jalaludin, A., Han, H., Yu, Q. & Powles, S. (2013) *Evolution in action: a double amino acid substitution in the EPSPS gene endows high-level glyphosate resistance*. IN: Global Herbicide Resistance Challenge 2013. Perth, Australia. Australian Herbicide Resistance Initiative. pp. 35.

James, C. (2012) *Global Status of Commercialized Biotech/GM Crops: 2012 Excutive Summary*.

Jasieniuk, M., Ahmad, R., Sherwood, A. M., Firestone, J. L., Perez-Jones, A., Lanini, W. T., Mallory-Smith, C. & Stednick, Z. (2008) Glyphosate-Resistant Italian Ryegrass (Lolium multiflorum) in California: Distribution, Response to Glyphosate, and Molecular Evidence for an Altered Target Enzyme. *Weed Science*, 56 (4): 496-502.

Jasieniuk, M., BruleBabel, A. L. & Morrison, I. N. (1996) The evolution and genetics of herbicide resistance in weeds. *Weed Science*, 44 (1): 176-193.

Johnson, W. G., Davis, V. M., Kruger, G. R. & Weller, S. C. (2009) Influence of glyphosate-resistant cropping systems on weed species shifts and glyphosate-resistant weed populations. *European Journal of Agronomy*, 31 (3): 162-172.

Jordan, N. (1999) Fitness effects of the triazine resistance mutation in Amaranthus hybridus : relative fi tness in maize and soyabean crops. *Weed Research*, 39 493-505.

Kaundun, S. S. (2013) *Evolution of resistance to acetyl CoA carboxylase-inhibiting herbicides*. IN: Global Herbicide Resistance Challenge 2013. Perth, Australia. Australian Herbicide Resistance Initiative. pp. 49.

Kaundun, S. S., Dale, R. P., Zelaya, I. A., Dinelli, G., Marotti, I., McIndoe, E. & Cairns, A. (2011) A Novel P106L Mutation in EPSPS and an Unknown Mechanism(s) Act Additively To Confer Resistance to Glyphosate in a South African Lolium rigidum Population. *Journal of Agricultural and Food Chemistry*, 59 (7): 3227-3233.

Kaundun, S. S., Zelaya, I., Dale, R. P., Lycett, A. J., Carter, P., Sharples, K. R. & McIndoe, E. (2008) Importance of the P106S Target-Site Mutation in Conferring Resistance to Glyphosate in a Goosegrass (Eleusine indica) Population from the Philippines. *Weed Science*, 56 (5): 637-646.

Kawakami, T., Strakosh, S. C., Zhen, Y. & Ungerer, M. C. (2010) Different scales of Ty1/copia-like retrotransposon proliferation in the genomes of three diploid hybrid sunflower species. *Heredity*, 104 (4): 341-350.

Keeley, P. E., Carter, C. H. & Thullen, R. J. (1987) Influence of planting date on growth of palmer amaranth (*Amaranthus palmeri*). *weed science*, 35 (2): 199-204.

Keller, L. F. & Waller, D. M. (2002) Inbreeding effects in wild populations. *Trends in Ecology & Evolution*, 17 (5): 230-241.

Keller, M. & Kollmann, J. (1999) Effects of seed provenance on germination of herbs for agricultural compensation sites. *Agriculture Ecosystems & Environment*, 72 (1): 87-99.

Kemper, B. & Derpsch, R. (1981) Results of studies made in 1978 and 1979 to control erosion by cover crops and no-tillage techniques in parana, Brazil. *Soil & Tillage Research*, 1 (3): 253-267.

Koch, A. L. (1983) The protein burden of lac operon products. *Journal Molecular Evolution* 19: 455–462.

Koger, C. H. & Reddy, K. N. (2005) Role of absorption and translocation in the mechanism of glyphosate resistance in horseweed (Conyza canadensis). *Weed Science*, 53 84-89.

Koricheva, J. (2002) Meta-analysis of sources of variation in fitness costs. *Ecology*, 83 (1): 176-190.

Lander, E. S. & Schork, N. J. (1994) Genetic dissection of complex traits. *Science*, 265 (5181): 2037-2048.

Lawrence, E. (2005) *Henderson's Dictionary of Biology*. Thirteenth edn. Essex: Pearson Education Limited.

Lee, L. J. & Ngim, J. (2000) A first report of glyphosate-resistant goosegrass (Eleusine indica (L) Gaertn) in Malaysia. *Pest Management Science*, 56 (4): 336-339.

Lee, R. M., Thimmapuram, J., Thinglum, K. a., Gong, G., Hernandez, A. G., Wright, C. L., Kim, R. W., Mikel, M. a. & Tranel, P. J. (2009) Sampling the Waterhemp (*Amaranthus tuberculatus*) Genome Using Pyrosequencing Technology. *Weed Science*, 57 (5): 463-469.

Lee, S. W. & Edlin, G. (1985) Expression of tetracycline resistance in pbr322 derivatives reduces the reproductive fitness of plasmid-containing *Escherichia coli*. *Gene*, 39 (2-3): 173-180.

Legere, A., Beckie, H. J., Stevenson, F. C. & Thomas, A. G. (2000) Survey of management practices affecting the occurrence of wild oat (Avena fatua) resistance to acetyl-CoA carboxylase inhibitors. *Weed Technology*, 14 (2): 366-376.

Legleiter, T. R. & Bradley, K. W. (2008) Glyphosate and Multiple Herbicide Resistance in Common Waterhemp (Amaranthus rudis) Populations from Missouri. *Weed Science*, 56 (4): 582-587.

Leon, R. G. & Owen, M. D. K. (2003) Regulation of weed seed dormancy through light and temperature interactions. *Weed Science*, 51 (5): 752-758.

Lermontova, I. & Grimm, B. (2000) Overexpression of plastidic protoporphyrinogen IX oxidase leads to resistance to the diphenyl-ether herbicide acifluorfen. *Plant Physiology*, 122 (1): 75-83.

L**ight, G. G., Mohammed, M. Y., Dotray, P. A., Chandler, J. M. & Wright, R. J.** (2011) Glyphosate-Resistant Common Waterhemp (Amaranthus rudis) Confirmed in Texas. *Weed Technology*, 25 (3): 480-485.

Lorraine-Colwill, D. F., Powles, S. B., Hawkes, T. R., Hollinshead, P. H., Warner, S. A. J. & Preston, C. (2002) Investigations into the mechanism of glyphosate resistance in Lolium rigidum. *Pesticide Biochemistry and Physiology*, 74 (2): 62-72.

Mackay, T. F. C. (1989) Transposable elements and fitness in *Drosophila melanogaster*. *Genome*, 31 (1): 284-295.

MacLean, R. C., Bell, G. & Rainey, P. B. (2004) The evolution of a pleiotropic fitness tradeoff in Pseudomonas fluorescens. *Proceedings of the National Academy of Sciences of the United States of America*, 101 (21): 8072-8077.

Maisnier-Patin, S. & Andersson, D. I. (2004) Adaptation to the deleterious effects of antimicrobial drug resistance mutations by compensatory evolution. *Research in Microbiology*, 155 (5): 360-369.

Mallory-Smith, C. & Zapiola, M. (2008) Gene flow from glyphosate-resistant crops. *Pest Management Science*, 64 (4): 428-440.

Mas, M. T., Verdú, A. M. C., Kruk, B. C., De Abelleyra, D., Guglielmini, A. C. & Satorre, E. H. (2010) Weed communities of transgenic glyphosate-tolerant soyabean crops in ex-pasture land in the southern Mesopotamic Pampas of Argentina. *Weed Research*, 50 320-330.

Mauch, F., Mauch-Mani, B., Gaille, C., Kull, B., Haas, D. & Reimmann, C. (2001) Manipulation of salicylate content in Arabidopsis thaliana by the expression of an engineered bacterial salicylate synthase. *Plant Journal*, 25 (1): 67-77.

Mauricio, R. (1998) Cost of Resistance to Natural Enemies Field Populations of the Annual Plant Arabidopsis thaliana. *The American Naturalist*, 151 (1): 20-28.

McCloskey, W. B. & Holt, J. S. (1990) Triazine resistance in *Senecio vulgaris* parental and nearly isonuclear backcrossed biotypes is correlated with reduced productivity. *Plant Physiology*, 92 (4): 954-962.

Medd, R. W., Van de Ven, R. J., Pickering, D. & Nordblom, T. (2001) Determination of environment-specific dose-response relationships for clodinafop-propargyl on *Avena spp. Weed Research*, 41 (4): 315-368.

Menchari, Y., Chauvel, B., Darmency, H. & Delye, C. (2008) Fitness costs associated with three mutant acetyl-coenzyme A carboxylase alleles endowing herbicide resistance in black-grass Alopecurus myosuroides. *Journal of Applied Ecology*, 45 (3): 939-947.

Menchari, Y., Chauvel, B., Darmency, H. & Délye, C. (2007) Fitness costs associated with three mutant acetyl-coenzyme A carboxylase alleles endowing herbicide resistance in black-grass Alopecurus myosuroides. *45*, 3 939-947.

Mendel, G. (1986) Experiments in a monastery garden. *American Zoologist*, 26 (3): 749-752.

Michitte, P., De Prado, R., Espinoza, N., Ruiz-Santaella, J. P. & Gauvrit, C. (2007) Mechanisms of resistance to glyphosate in a Ryegrass (*Lolium multiflorum*) biotype from Chile. *Weed Science*, 55 (5): 435-440.

Mohseni-Moghadam, M., Schroeder, J. & Ashigh, J. (2013) Mechanism of resistance and inheritance in glyphosate resistant Palmer amaranth (*Amaranthus palmeri*) populations from New Mexico, USA. Weed Science In-Press. *Weed Science*, In-Press

Mortimer, A. & Maxwell, B. (1994) Selection for herbicide resistance. In: Herbicide Resistance in Plants: Biology and Biochemistry (eds SB Powles & Hotum JAM), 1–25. CRC Press, Boca Raton, FL, USA.

Moss, S. R. (2010) Herbicide resistance in northern Europe: current status; future issues. in: Proceedings, Pan-American Weed Resistance Conference, Miami, FL, pp. 44-45.

Moss, S. R. (2002) *Weed Management Handbook*. 9th edn. Oxford: Blackwell Science Ltd.

Mueller, T. C., Mitchell, P. D., Young, B. G. & Culpepper, A. S. (2005) Proactive Versus Reactive Management of Glyphosate-Resistant or -Tolerant Weeds. *Weed technology*, 19 924-933.

Murray, M. J. (1940) The genetics of sex determination in the family Amaranthaceae. *Genetics*, 25 409–431.

Nandula, V., Ray, J. D., Ribeiro, D., Pan, Z. & Reddy, K. (2013) Glyphosate Resistance in Tall Waterhemp (*Amaranthus tuberculatus*) from Mississippi is due to both Altered Target Site and Non-Target Site Mechanisms. *Weed Science*, In-Press

Nandula, V. K., Reddy, K. N., Poston, D. H., Rimando, A. M. & Duke, S. O. (2008) Glyphosate tolerance mechanism in Italian ryegrass (*Lolium multiflorum*) from Mississippi. *Weed Science*, 56 (3): 344-349.

Naylor, R. E. L. & Drummond, C. (2002) *Weed Management Handbook*. 9th edn. Oxford: Blackwell Science Ltd.

Neve, P. (2007) Challenges for herbicide resistance evolution and management: 50 years after Harper. *Weed Research*, 47 (5): 365-369.

Neve, P. (2008) Simulation modelling to understand the evolution and management of glyphosate resistant in weeds. *Pest Management Science*, 64 (4): 392-401.

Neve, P., Diggle, A. J., Smithà, F. P. & Powles, S. B. (2003) Simulating evolution of glyphosate resistance in Lolium rigidum II : past , present and future glyphosate use in Australian cropping. *Weed Research*, 43 418-427.

Neve, P., Norsworthy, J. K., Smith, K. L. & Zelaya, I. A. (2010) Modelling evolution and management of glyphosate resistance in Amaranthus palmeri. *Weed Research*, 51 (2): 99-112.

Neve, P., Norsworthy, J. K., Smith, K. L. & Zelaya, I. A. (2011) Modeling Glyphosate Resistance Management Strategies for Palmer Amaranth (Amaranthus palmeri) in Cotton. *Weed Technology*, 25 (3): 475-487.

Neve, P. & Powles, S. (2005) Recurrent selection with reduced herbicide rates results in the rapid evolution of herbicide resistance in Lolium rigidum. *Theoretical and applied genetics.*, 110 (6): 1154-1166.

Neve, P., Vila-aiub, M. & Roux, F. (2009) Evolutionary-thinking in agricultural weed management. *New Phytologist*, (Review): 1-11.

Ng, C. H., Wickneswari, R., Salmijah, S., Teng, Y. T. & Ismail, B. S. (2003) Gene polymorphisms in glyphosate-resistant and -susceptible biotypes of Eleusine indica from Malaysia. *Weed Research*, 43 (2): 108-115.

Nguyen, T. N. M., Phan, Q. G., Duong, L. P., Bertrand, K. P. & Lenski, R. E. (1989) Effects of carriage and expression of the tn10 tetracycline-resistance operon on the fitness of *Escherichia coli*-k12. *Molecular Biology and Evolution*, 6 (3): 213-225.

Norsworthy, J. (2013) *Glyphosate-resistant Palmer amaranth (Amaranthus palmeri) in southern USA row-crop production: impact and current management strategies*. IN: Global Herbicide Resistance Challenge 2013. Perth, Australia. Australian Herbicide Resistance Initiative. pp. 92.

O'Neill, R. J. W., O'Neill, M. J. & Graves, J. A. M. (1998) Undermethylation associated with retroelement activation and chromosome remodelling in an interspecific mammalian hybrid. *Nature*, 393 (6680): 68-72.

Oerke, E. C. (2006) Crop losses to pests. *Journal of Agricultural Science*, 144 31-43.

Orr, H. A. (2009) Fitness and its role in evolutionary genetics. *Nature reviews. Genetics*, 10 (8): 531-539.

Orr, H. A. & Betancourt, A. J. (2001) Haldane's sieve and adaptation from the standing genetic variation. *Genetics*, 157 (2): 875-884.

Owen, M. (1997) *Weed and crop resistance to herbicides*. London: Kluwer Academic Publishers.

Owen, M. D., **Shaw, D. R.**, **Weller, S. C.**, **Dixon, P. M.**, **Young**, **B. G.**, **Jordan**, **D. L.** & **Wilson, R. G.** (2013) The Benchmark Study – a field scale project demonstrating the sustainability of glyphosate-based crop production. IN: Global Herbicide Resistance Challenge 2013. Perth, Australia. Australian Herbicide Resistance Initiative. pp. 33.

Owen, M. D. K. (2001) *World maize/soybean and herbicide resistance, in Herbicide resistance and world grains*. Boca Raton: CRC Press.

Owen, M. D. K. & Zelaya, I. A. (2005) Herbicide-resistant crops and weed resistance to herbicides. *Pest Management Science*, 61 (3): 301-311.

Padgette, S. R., Re, D. B., Gasser, C. S., Eichholtz, D. a., Frazier, R. B., Hironaka, C. M., Levine, E. B., Shah, D. M., Fraley, R. T. & Kishore, G. M. (1991) Site-directed mutagenesis of a conserved region of the 5-enolpyruvylshikimate-3-phosphate synthase active site. *The Journal of biological chemistry*, 226 (33): 22364-22369.

Paris, M., Roux, F., Berard, A. & Reboud, X. (2008) The effects of the genetic background on herbicide resistance fitness cost and its associated dominance in Arabidopsis thaliana. *Heredity*, 101 (6): 499-506.

Paris, M., Roux, F., Bérard, A. & Reboud, X. (2006) The effects of the genetic background on herbicide resistance fitness cost and its associated dominance in Arabidopsis thaliana. *Heredity*, 101 (6): 499-506.

Park, S. E., Benjamin, L. R. & Watkinson, A. R. (2003) The theory and application of plant competition models: an agronomic perspective. *Annals of Botany*, 92 (6): 741-748.

Parker, M. A. (1992) Disease and plant population genetic structure, in: **Plant Resistance to Herbivores and Pathogens:** Ecology, Evolution, and Genetics Robert S. Fritz, Ellen L. Simms University of Chicago Press,. Chicago, pp. 345

Patzoldt, W. L., Tranel, P. J. & Hager, A. G. (2002) Variable herbicide responses among Illinois waterhemp (*Amaranthus rudis* and *A. tuberculatus*) populations. *Crop Protection*, 21 (9): 707-712.

Patzoldt, W. L., Tranel, P. J. & Hager, A. G. (2005) A waterhemp (*Amaranthus tuberculatus*) biotype with multiple resistance across three herbicide sites of action. *Weed Science*, 53 (1): 30-36.

Pedersen, B., Neve, P., Andreasen, C. & Powles, S. (2007) Ecological fitness of a glyphosate-resistant *Lolium rigidum* population: Growth and seed production along a competition gradient. *Basic and Applied Ecology*, 8 (3): 258-268.

Perez-Jones, A., Park, K.-W., Polge, N., Colquhoun, J. & Mallory-Smith, C. A. (2007) Investigating the mechanisms of glyphosate resistance in *Lolium multiflorum*. *Planta*, 226 (2): 395-404.

Plowman, A. B. & Richards, A. J. (1997) The effect of light and temperature on competition between atrazine susceptible and resistant Brassica rapa. *Annals of Botany*, 80 (5): 583-590.

Powles, S. B. (2008) Evolved glyphosate-resistant weeds around the world: lessons to be learnt. *Pest management science*, 365 360-365.

Powles, S. B., Lorraine-Colwill, D. F., Dellow, J. J. & Preston, C. (1998) Evolved resistance to glyphosate in rigid ryegrass (*Lolium rigidum*) in Australia. *Weed Science*, 46 (5): 604-607.

Powles, S. B. & Preston, C. (2006) Evolved Glyphosate Resistance in Plants: Biochemical and Genetic Basis of Resistance. *Weed Technology*, 20 (2): 282-289.

Powles, S. B. & Yu, Q. (2010) Evolution in action: plants resistant to herbicides. *Annual review of plant biology*, 61 317-347.

Pratley, J., Urwin, N., Stanton, R., Baines, P., Broster, J., Cullis, K., Schafer, D., Bohn, J. & Krueger, R. (1999) Resistance to glyphosate in Lolium rigidum. I. Bioevaluation. *Weed Science*, 47 (4): 405-411.

Pratt, D. B. & Clark, L. G. (2001) Amaranthus rudis and *A. tuberculatus*, One Species or Two? *Journal of the Torrey Botanical Society*, 128 (3): 282-296.

Preston, C. & Wakelin, A. M. (2008) Resistance to glyphosate from altered herbicide translocation patterns. *Pest Management Science*, 64 (4): 372-376.

Preston, C., Wakelin, A. M., Dolman, F. C., Bostamam, Y. & Boutsalis, P. (2009) A Decade of Glyphosate-Resistant Lolium around the World: Mechanisms, Genes, Fitness, and Agronomic Management. *Weed Science*, 57 (4): 435-441.

Price, A. J., Balkcom, K. S., Culpepper, S. A., Kelton, J. A., Nichols, R. L. & Schomberg, H. (2011) Glyphosate-resistant Palmer amaranth: A threat to conservation tillage. *Journal of Soil and Water Conservation*, 66 (4): 265-275.

Primack, R. B. & Kang, H. (1989) Measuring fitness and natural selection in wild plant populations. *Johnston, R. F. (Ed.). Annual Review of Ecology and Systematics, Vol. 20. Ix+477p. Annual Reviews Inc.: Palo Alto, California, USA. Illus. Maps*, 367-396.

Purrington, C. B. (2000) Costs of resistance. *Current opinion in plant biology*, 3 (4): 305-308.

Purrington, C. B. & Bergelson, J. (1997) Fitness consequences of genetically engineered herbicide and antibiotic resistance in Arabidopsis thaliana. *Genetics*, 145 (3): 807-814.

Reddy, K. N., Rimando, A. M., Duke, S. O. & Nandula, V. K. (2008) Aminomethylphosphonic acid accumulation in plant species treated with glyphosate. *Journal of Agricultural and Food Chemistry*, 56 (6): 2125-2130.

Reeves, D. W. (1997) The role of soil organic matter in maintaining soil quality in continuous cropping systems. *Soil & Tillage Research*, 43 (1-2): 131-167.

Ritz, C. & Streibi, J. C. (2012) Dose response curves and other nonlinear curves in Weed Science and Ecotoxicology with the add-on package drc in R.

Ritz, C. & Streibig, J. C. (2005) Bioassay analysis using R. *Journal of Statistical Software*, 12

Roux, F., Camilleri, C., Berard, A. & Reboud, X. (2005a) Multigenerational versus single generation studies to estimate herbicide resistance fitness cost in Arabidopsis thaliana. *Evolution*, 59 (10): 2264-2269.

Roux, F., Camilleri, C., Giancola, S., Brunel, D. & Reboud, X. (2005b) Epistatic interactions among herbicide resistances in Arabidopsis thaliana: the fitness cost of multiresistance. *Genetics Society of America*, 171 (3): 1277-1288.

Roux, F., Gasquez, J. & Reboud, X. (2004) The Dominance of the Herbicide Resistance Cost in Several Arabidopsis thaliana Mutant Lines. *Genetics Society of America*, 460: 449-460.

Roux, F., Giancola, S., Durand, S. & Reboud, X. (2006) Building of an experimental cline with Arabidopsis thaliana to estimate herbicide fitness cost. *Genetics*, 173 (2): 1023-1031.

Roux, F., Matejicek, A. & Reboud, X. (2005c) Response of Arabidopsis thaliana to 22 ALS inhibitors: baseline toxicity and cross-resistance of csr1-1 and csr1-2 resistant mutants. *Weed Research*, 45 (3): 220-227.

Roux, F., Paris, M. & Reboud, X. (2008) Delaying weed adaptation to herbicide by environmental heterogeneity : a simulation approach. *Pest Management Science*, 64: 16-29.

Ryan, G. F. (1970) Resistance of common groundsel to simazine and atrazine. *Weed Science*, 18 (5): 614-616.

Rüegg, W. T., Quadranti, M. & Zoschke, A. (2007) Herbicide research and development: challenges and opportunities. *Weed Research*, 47 (4): 271-275.

Samsel, A. & Seneff, S. (2013) Glyphosate's Suppression of Cytochrome P450 Enzymes and Amino Acid Biosynthesis by the Gut Microbiome: Pathways to Modern Diseases. *Entropy*, 15 (4): 1416-1463.

Samson, D. A. & Werk, K. S. (1986) Size-dependent effects in the analysis of reproductive effort in plants. *American Naturalist*, 127 (5): 667-680.

Saroha, M. K., Sridhar, P. & Malik, V. S. (1998) Glyphosate-tolerant crops: Genes and enzymes. *Journal of Plant Biochemistry and Biotechnology*, 7 (2): 65-72.

Sauer, J. (1957) Recent migration and evolution of the dioecious amaranths. *Evolution*, 11 11–31.

Sauer, J. (1972) The dioecious amaranths: a new species name and major range extensions. *Madroño*, 21 425–434.

Schmitt, J., Eccleston, J. & Ehrhardt, D. W. (1987) Dominance and suppression, size-dependent growth and self-thinning in a natural *Impatiens capensis* population. *Journal of Ecology*, 75 (3): 651-665.

Schwinning, S. & Weiner, J. (1998) Mechanisms determining the degree of size asymmetry in competition among plants. *Oecologia*, 113 (4): 447-455.

Schönbrunn, E., Eschenburg, S., Shuttleworth, W. A., Schloss, J. V., Amrhein, N., Evans, J. N. & Kabsch, W. (2001) Interaction of the herbicide glyphosate with its target enzyme 5-enolpyruvylshikimate 3-phosphate synthase in atomic detail. *Proceedings of the National Academy of Sciences of the United States of America*, 98 (4): 1376-1380.

Seefeldt, S. S., Jensen, J. E. & Fuerst, E. P. (1995) Log-logistic analysis of herbicide dose response relationships. *Weed Technology*, 9 (2): 218-227.

Shaner, D. (2010) *Glyphosate resistance in crops and weeds: History, Development, and Management*. Hoboken, NJ, USA: John Wiley & Sons, Inc.

Shaner, D. L., Nadler-Hassar, T., Henry, W. B. & Koger, C. H. (2005) A rapid in vivo shikimate accumulation assay with excised leaf discs. *Weed Science*, 53 (6): 769-774.

Shaw, D. R., Arnold, J. C., Snipes, C. E., Laughlin, D. H. & Mills, J. A. (2001) Comparison of glyphosate-resistant and nontransgenic soybean (Glycine max) herbicide systems. *Weed Technology*, 15 (4): 676-685.

Silvertown, J. & Charlesworth, D. (2001) *Introduction to plant population biology*. Oxford: Blackwell Science.

Simarmata, M. & Penner, D. (2008) The basis for glyphosate resistance in rigid ryegrass (Lolium rigidum) from California. *Weed Science*, 56 (2): 181-188.

Simonsen, L., Fomsgaard, I. S., Svensmark, B. & Spliid, N. H. (2008) Fate and availability of glyphosate and AMPA in agricultural soil. *Journal of Environmental Science and Health Part B-Pesticides Food Contaminants and Agricultural Wastes*, 43 (5): 365-375.

Singh, B. K., Siehl, D. L. & Connelly, J. A. (1991) Shikimate pathway why does it mean so much to so many? *Miflin, B. J. (Ed.). Oxford Surveys of Plant Molecular and Cell Biology, Vol. 7. Vii 334p. Oxford University Press: Oxford, England, Uk; New York, New York, USA. Illus. Paper*,

Smeda, R. J. & Schuster, C. L. (2002) Differential sensitivity to glyphosate among biotypes of common waterhemp (*Amaranthusrudis Sauer*). *13th Australian Weeds Conference. Perth, Australia*. *Council of Australian Weed Science Societies*, pp. 642.

Smith, D. A. & Hallett, S. G. (2006) Variable Response of Common Waterhemp (*Amaranthus rudis*) Populations and Individuals to Glyphosate. *Weed Technology*, 20 (2): 466-471.

Sprague, C. L., Stoller, E. W. & Wax, L. M. (1997) Response of an acetolactate synthase (ALS)-resistant biotype of *Amaranthus rudis* to selected ALS-inhibiting and alternative herbicides. *Weed Research*, 37 (2): 93-101.

Stalker, D. M., Hiatt, W. R. & Comai, L. (1985) A single amino-acid substitution in the enzyme 5-enolpyruvylshikimate-3-phosphate synthase confers resistance to the herbicide glyphosate. *Journal of Biological Chemistry*, 260 (8): 4724-4728.

Steckel, L. E. (2007) The Dioecious Amaranthus spp.: Here to Stay. *Weed Technology*, 21 (2): 567-570.

Steckel, L. E. & Sprague, C. L. (2004) Late-season common waterhemp (Amaranthus rudis) interference in narrow- and wide-row soybean. *Weed Technology*, 18 (4): 947-952.

Steckel, L. E., Sprague, C. L., Stoller, E. W. & Wax, L. M. (2004) Temperature effects on germination of nine Amaranthus species. *Weed Science*, 52 (2): 217-221.

Steckel, L. E., Sprague, C. L., Stoller, E. W., Wax, L. M. & Simmons, F. W. (2007) Tillage, cropping system, and soil depth effects on common waterhemp (Amaranthus rudis) seed-bank persistence. *Weed Science*, 55 (3): 235-239.

Stefano, J. E., Ackerson, J. W. & Gralla, J. D. (1980) Alterations in 2 conserved regions of promoter sequence lead to altered rates of polymerase binding and levels of gene-expression. *Nucleic Acids Research*, 8 (12): 2709-2723.

Steinau, A. N., Skinner, D. Z. & Steinau, M. (2003) Mechanism of extreme genetic recombination in weedy Amaranthus hybrids. *Weed Science*, 51 (5): 696-701.

Stoebel, D. M., Dean, A. M., & Dykhuizen, D. E. (2008) The Cost of Expression of *Escherichia coli* lac Operon Proteins Is in the Process, Not in the Products. *Genetics Society of America*, 178: 1653–1660.

Strauss, S. Y. & Agrawal, A. A. (1999) The ecology and evolution of plant tolerance to herbivory. *Trends in ecology & evolution*, 14 (5): 179-185.

Tardif, F. J. & Powles, S. B. (1999) Effect of malathion on resistance to soil-applied herbicides in a population of rigid ryegrass (Lolium rigidum). *Weed Science*, 47 (3): 258-261.

Tardif, F. J., Rajcan, I. & Costea, M. (2006) A mutation in the herbicide target site acetohydroxyacid synthase produces morphological and structural alterations and reduces fitness in Amaranthus powellii. *The New phytologist*, 169 (2): 251-264.

Tatnell, L. V., Ginsburg, D., Moss, S. R., Marshall, R. & Clarke, J. H. (2007) *A review of broad-leaved weed resistance 2006- 2007. As part of project PS2709 funded by Defra-PSD*.

R Development Core Team (2013). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/.

Thai, H. N., Malone, J., Boutsalis, P. & Preston, C. (2012) *Glyphosate resistance in barnyard grass (Echinochloa colona)*. IN: Eldershaw, V. Eighteenth Australasian Weeds Conference. Weed Society of Victoria Inc., 237- 240.

Tharp, B. E. & Kells, J. J. (2001) Effect of glufosinate-resistant corn (Zea mays) population and row spacing on light interception, corn yield, and common lambsquarters (Chenopodium album) growth. *Weed Technology*, 15 (3): 413-418.

Tranel, P. J., Lee, R. M., Bell, M. S., Singh, S., Walter, J. R. & Bradley, K. W. (2006) What we know (and don't know) about glyphosate resistance in waterhemp. *North Central Weed Science Society Proceedings*, 61: 100

Tranel, P. J., Riggins, C. W., Bell, M. S. & Hager, A. G. (2011) Herbicide Resistances in *Amaranthus tuberculatus*: A Call for New Options. *Journal of Agricultural and Food Chemistry*, 59 (11): 5808-5812.

Trucco, F., Tatum, T., Rayburn, a. L. & Tranel, P. J. (2009) Out of the swamp: unidirectional hybridization with weedy species may explain the prevalence of *Amaranthus tuberculatus* as a weed. *The New phytologist*, 184 (4): 819-827.

Trucco, F., Zheng, D., Woodyard, A. J., Walter, J. R., Tatum, T. C., Rayburn, A. L. & Tranel, P. J. (2007) Nonhybrid progeny from crosses of dioecious amaranths: Implications for gene-flow research. *Weed Science*, 55 (2): 119-122.

United Nations, Division of economic and social affairs (2009) *Division World Population Prospects: The 2008 Revision*[online]. http://esa.un.org/wpp/ (Acessed:**November 30, 2012**).

Uscanga-Mortera, E., Clay, S. A., Forcella, F. & Gunsolus, J. (2007) Common Waterhemp Growth and Fecundity as Influenced by Emergence Date and Competing Crop. *Agronomy Journal*, 99 (5): 1265-1270.

USDA (2011) *Minnesota Agricultural Statistics. Crop county estimates. corn: Acreage, Yield, and Production, by County and District, Minnesota, 2009-2010*. [online] Available from: http://www.nass.usda.gov/Statistics_by_State/Minnesota/Publications/Annual_Statistical _Bulletin/2012/26-27.pdf (**Acessed August 30, 2013**).

USDA (2012) Genetically engineered varieties of corn, upland cotton, and soybeans, by State and for the Unites States, 2000-2012**.** [Online Database] Available from: www.ers.usda.gov (Acessed **August 30, 2013**).

Van Dam, N. M. & Baldwin, I. T. (2001) Competition mediates costs of jasmonate-induced defences, nitrogen acquisition and transgenerational plasticity in Nicotiana attenuata. *Functional Ecology*, 15 (3): 406-415.

VanGessel, M. J. (2001) Glyphosate-resistant horseweed from Delaware. *Weed Science*, 49 (6): 703-705.

Vencill, W. K., Nichols, R. L., Webster, T. M., Soteres, J. K., Mallory-Smith, C., Burgos, N. R., Johnson, W. G. & McClelland, M. R. (2012) Herbicide Resistance: Toward an Understanding of Resistance Development and the Impact of Herbicide-Resistant Crops. *Weed Science*, 60: 2-30.

Vila-Aiub, M. M., Balbi, M.C, Gundel, P. E., Ghersa, C. M. & Powles, S. B. (*2007*) Evolution of Glyphosate-Resistant Johnsongrass (*Sorghum halepense*) in Glyphosate-Resistant Soybean. *Weed Science*, 55(6): 566-571.

Vila-aiub, M. M., Neve, P. & Powles, S. B. (2005) Resistance cost of a cytochrome P450 herbicide metabolism mechanism but not an ACCase target site mutation in a multiple resistant Lolium rigidum population. *New Phytologist*, 176: 787-796.

Vila-Aiub, M. M., Neve, P. & Powles, S. B. (2009a) Fitness costs associated with evolved herbicide resistance alleles in plants. *New Phytologist*, 184 (4): 751-567.

Vila-Aiub, M. M., Neve, P. & Powles, S. B. (2009b) Evidence for an ecological cost of enhanced herbicide metabolism in Lolium rigidum. *Journal of Ecology*, 97 (4): 772-780.

Vila-Aiub, M. M., Neve, P. & Roux, F. (2011) A unified approach to the estimation and interpretation of resistance costs in plants. *Heredity*, 107 (5): 386-894.

Vila-Aiub, M. M., Neve, P., Steadman, K. J. & Powles, S. B. (2005) Ecological fitness of a multiple herbicide-resistant Lolium rigidum population: dynamics of seed germination and seedling emergence of resistant and susceptible phenotypes. *Journal of Applied Ecology*, 42 (2): 288-298.

Vila-Aiub, M. M., Vidal, R. A., Balbi, M. C., Gundel, P. E., Trucco, F. & Ghersa, C. M. (2008) Glyphosate-resistant weeds of South American cropping systems: an overview. *Pest Management Science*, 64 (4): 366-371.

Wagner, A. (2005) Energy Constraints on the Evolution of Gene Expression. *Society for Molecular Biology and Evolution*, 22(6): 1365–1374.

Wakelin, A. M. & Preston, C. (2006) A target-site mutation is present in a glyphosate-resistant Lolium rigidum population. *Weed research*, 46: 432-440.

Wall, R. & Begon, M. (1985) Competition and fitness. *Oikos*, 44 (2): 356-360.

Wang, T., Picard, J. C., Tian, X. & Darmency, H. (2010) A herbicide-resistant ACCase 1781 Setaria mutant shows higher fitness than wild type. *Heredity*, 105 (4): 394-400.

Wang, W., Xia, H., Yang, X., Xu, T., Si, H. J., Cai, X. X., Wang, F., Su, J., Snow, A. A. & Lu, B. R. (2013) A novel 5-enolpyruvoylshikimate-3-phosphate (EPSP) synthase transgene for glyphosate resistance stimulates growth and fecundity in weedy rice (Oryza sativa) without herbicide. *New Phytologist*, Published online

Ward, S. & Giacomini, D. (2013) *Estimating fitness costs of glyphosate resistance: Palmer amaranth (Amaranthus palmeri) as a case study*. IN: Global Herbicide Resistance Challenge 2013. Perth, Australia. Australian Herbicide Resistance Initiative. pp. 79

Warwick, S. I. & Black, L. D. (1994) Relative fitnes of herbicide-resistant and susceptible biotypes of weeds. *Phytoprotection*, 75 (4): 37-49.

Wax, L. M. (1995) *Pigweeds of the Midwest: distribution, importance and management.* IN: Proceedings of the Integrated Crop Management Conference. Iowa State University, 239-242.

Weaver, M. A., Krutz, L. J., Zablotowicz, R. M. & Reddy, K. N. (2007) Effects of glyphosate on soil microbial communities and its mineralization in a Mississippi soil. *Pest management science*, 393: 388-393.

Weiner, J. (1982) A neighborhood model of annual-plant interference. *Ecology*, 63 (5): 1237-1241.

Weiner, J. (1990) Asymmetric competition in plant-populations. *Trends in Ecology & Evolution*, 5 (11): 360-364.

Weiner, J., Stoll, P., Muller-Landau, H. & Jasentuliyana, A. (2001) The effects of density, spatial pattern, and competitive symmetry on size variation in simulated plant populations. *American Naturalist*, 158 (4): 438-450.

Wessler, S. R., Bureau, T. E. & White, S. E. (1995) LTR-retrotransposons and MITES - important players in the evolution of plant genomes. *Current Opinion in Genetics & Development*, 5 (6): 814-821.

Wetzel, D. K., Horak, M. J., Skinner, D. Z. & Kulakow, P. A. (1999) Transferal of herbicide resistance traits from Amaranthus palmeri to Amaranthus rudis. *Weed Science*, 47 (5): 538-543.

Widholm, J. M., Chinnala, A. R., Ryu, J. H., Song, H. S., Eggett, T. & Brotherton, J. E. (2001) Glyphosate selection of gene amplification in suspension cultures of 3 plant species. *Physiologia Plantarum*, 112 (4): 540-545.

Williams, G. M., Kroes, R. & Munro, I. C. (2000) Safety evaluation and risk assessment of the herbicide Roundup and its active ingredient, glyphosate, for humans. *Regulatory Toxicology and Pharmacology*, 31 (2): 117-165.

Ye, S. (2011) *Minnesota Crops Profile*. Minnesota Department of Agriculture. [online] Available from: http://www.mda.state.mn.us/food/business/~/media/Files/food/business /economics/mncropsprofile.ashx (Acessed **August 30, 2013**).

Young, B. G. (2006) Changes in Herbicide Use Patterns and Production Practices Resulting from Glyphosate-Resistant Crops. *Weed Technology*, 20 301-307.

Young, N. D. & Tanksley, S. D. (1989) RFLP analysis of the size of chromosomal segments retained around the tm-2 locus of tomato during backcross breeding. *Theoretical and Applied Genetics*, 77 (3): 353-359.

Yu, Q. (2013) *Resistance to AHAS-inhibiting herbicides: the current understanding*. IN: Global Herbicide Resistance Challenge 2013. Perth, Australia. Australian Herbicide Resistance Initiative. pp. 48.

Yu, Q., Cairns, A. & Powles, S. (2007) Glyphosate, paraquat and ACCase multiple herbicide resistance evolved in a Lolium rigidum biotype. *Planta*, 225 (2): 499-513.

Zelaya, I. A. & Owen, M. D. K. (2000) Differential response of common waterhemp (*Amaranthus rudis*) to glyphosate in Iowa. *Weed Sci Soc Am*, 40: 62-63.

Zelaya, I. A. & Owen, M. D. K. (2002) *Amaranthus tuberculatus (Mq. ex DC) J.D.Sauer: potential for selection of glyphosate resistance*. IN: Jacobb, H. S., Dodd, J. & Moore, J. H. *Thirteenth Australian Weeds Conference*. Coucil Australian of Weed Science Societies, pp 630-633.

Zelaya, I. A. & Owen, M. D. K. (2005) Differential response of *Amaranthus tuberculatus* (Moq ex DC) JD Sauer to glyphosate. *Pest management science*, 61 (10): 936-950.

Zelaya, I. A., Owen, M. D. K. & VanGessel, M. J. (2004) Inheritance of evolved glyphosate resistance in *Conyza canadensis* (L.) Cronq. *Theoretical and Applied Genetics*, 110 58-70.

Zhang, N. Y., Linscombe, S. & Oard, J. (2003) Out-crossing frequency and genetic analysis of hybrids between transgenic glufosinate herbicide-resistant rice and the weed, red rice. *Euphytica*, 130 (1): 35-45.

Zhou, H., Arrowsmith, J. W., Fromm, M. E., Hironaka, C. M., Taylor, M. L., Rodriguez, D., Pajeau, M. E., Brown, S. M., Santino, C. G. & Fry, J. E. (1995) Glyphosate-tolerant *CP4* and *GOX* genes as a selectable marker in wheat transformation. *Plant Cell Reports*, 15 (3-4): 159-163.